

EXHIBIT

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Resume / Curriculum Vitae

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SUMMARY:

A research scientist, department head, and vice president with 16 years of experience in drug-discovery and development. Accomplishments include establishing and leading discipline-based research groups (pharmacology, neurochemistry, imaging, high throughput screening); 2) leading drug-discovery programs (target selection through clinical candidate evaluation) for a variety of disease targets (migraine, neuropathic pain and neuropsychiatric disorders); 3) Head of highly productive research-based drug discovery organization. responsible for advancing hits to leads to drug-candidates through preclinical evaluation and clinical testing.. Member executive management team of ACADIA Pharmaceuticals.

Academic experience includes postgraduate training and appointment as a research assistant professor at Duke University. Accomplishments in academic settings include: establishing a productive research laboratory; successfully obtaining extramural funding (R29); publication of peer-reviewed manuscripts in quality journals and; the training and placement of post-doctoral fellows. For 5 years I taught a course in Molecular Pharmacology and drug-discovery for the University of California Berkeley extension service.

Other accomplishments, interests and endeavors include:

- Family – married twenty five years with two children;
- Commercial rated pilot (glider and power) with a passion for aerobatics;
- Former senior member of the US National Ski Patrol

EDUCATION:

B.Sc. (Physiology) 1980, Michigan State University, East Lansing, Michigan.

Ph.D. (Pharmacology and Toxicology) 1983, University of Arizona, Tucson, Arizona.

PROFESSIONAL EXPERIENCE (Academic positions):

Undergraduate teaching assistant (Physics department) 1979-1980, Michigan State University, East Lansing, Michigan.

Graduate teaching assistant (Department of Medicine) and lecturer (Departments of Pharmacology and Anatomy) 1981-1982. University of Arizona, health sciences center, Tucson, Arizona.

Research Associate, (Department of Medicine, Division of Neurology) 1983-1987, Duke University, Durham, North Carolina (with a second appointment as a Research Associate at the Veterans Administration Medical Center, Durham, North Carolina).

Research Assistant Professor, (Department of Medicine, Division of Neurology) 1987-1991, Duke University, Durham, North Carolina (with a second appointment as a Research Pharmacologist at the Veterans Administration Medical Center, Durham, NC).

Instructor and course co-coordinator (Molecular Pharmacology), 1997 – 2005, University of California, Berkeley extension service.

PROFESSIONAL EXPERIENCE (Industrial positions):

Staff Researcher II, Syntex Research (Institute of Pharmacology), Palo Alto California 1991-1995.

Principal Scientist, Roche Bioscience (Neurobiology unit), Palo Alto, California 1995 - 2000

Department head / Senior Scientist Roche Palo Alto, Palo Alto California 2000 – 2005

Vice president Biosciences ACADIA pharmaceuticals 2005-current

GRANTS AND AWARDS:

Department of Health and Human Services Individual National Research Award (1985-1986).

Veterans Administration Merit Review Research Award (1989-1991).

Department of Health and Human Services First Independent Research Support and Transition Award (R29) (1989-1995).

PUBLICATIONS:

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- K.P. Zeitz, N. Guy, A.B. Malmberg, S. Dirajlal, W.J., Martin, L. Sun, D.W. Bonhaus, C.L. Stucky, D. Julius, A.I. Basbaum. The 5-HT3 subtype of serotonin receptor contributes to nociceptive processing via a novel subset of myelinated and unmyelinated nociceptors. *Journal of Neuroscience*. 22: 1010-1019, 2002.
- R.E. Wilcox, J.E. Ragan, R.S. Pearlman, M.Y-K. Brusniak, R.M. Eglen, D.W. Bonhaus, T.E. Tenner, Jr. and J.D. Miller. High-affinity interactions of ligands at recombinant guinea pig 5HT7 receptors. *Journal of Computer-aided Molecular Design*. 15: 883–909, 2001.
- Michele T. Taylor and Douglas W. Bonhaus. Allosteric modulation of [3H]gabapentin binding by the calcium channel ligands, ruthenium red, spermine and MgCl₂ *Neuropharmacology* 397: 1267-1273 2000.
- Douglas W. Bonhaus, Lee A. Flippin, Robert J. Greenhouse, Saul Jaime, Cindy Rocha, Mark Dawson, Kristine Van Natta, L.K. Chang, Tess Pulido-Rios, Andrea Webber, Richard M. Eglen and Graeme R. Martin RS-127445: a selective, high affinity, orally bioavailable 5-HT_{2B} receptor antagonist. *British Journal of Pharmacology* 129: 1075-1082 1999
- D.W. Bonhaus, L.K. Chang, Z. Cao, L. Wong, T. Pulido-Rios, A. Webber, E. Leung, L.A. Flippin, R.M. Eglen and G. R. Martin. RS-127445, a selective 5-HT_{2B} receptor

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S.J. MacLennan, P.H. Reynen, J. Kwan and D.W. Bonhaus Evidence for inverse agonism of SR141716A at human recombinant cannabinoid CB₁ and CB₂ receptors British Journal of Pharmacology 124: 619-622, 1998.

J.R. Jasper, J.D. Lesnick, L.K. Chang, S.S. Yamanishi, T.K. Chang, S. Hsu, D.A. Daunt, D.W. Bonhaus and R.M. Eglen Ligand efficacy and potency at recombinant Alpha₂ adrenergic receptors assessed through agonist-mediated [³⁵S]GTPγS binding. Biochemical Pharmacology 55: 1035-1043, 1998

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D.W. Bonhaus, J. Berger, N. Adham, T.A. Branchek, S.A.O. Hsu, D.N. Loury, E. Leung, E.H.F. Wong, R.D. Clark and R.M. Eglen. [³H]RS 57639, a high affinity, selective 5-HT₄ receptor agonist, specifically labels guinea pig striatal and rat cloned (5-HT_{4S} and 5-HT_{4L}) receptors. Neuropharmacology 36 671-679, 1997.

Douglas W. Bonhaus, Klaus K. Weinhardt, Mike Taylor, Andrea DeSouza, Patty M. McNeeley, Krystine Szczepanski, David J. Fontana, Lee A. Flippin, and Richard M. Eglen. RS-102221: a novel high affinity and selective, 5-HT_{2C} receptor antagonist Neuropharmacology 36: 621-629 1997.

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Elizabeth A. Kunysz, Melissa Lukes, and Douglas W. Bonhaus: Bar-code technology and a centralized database: key components in a radioligand binding high throughput screening program. In High Throughput Screening: The Discovery of Bioactive

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Antony P.D.W. Ford, Nicolas F. Arrendondo, David R. Blue Jr., Douglas W. Bonhaus, Jeffery Jasper, M. Shannon Kava, John Lesnick, Jurg R. Pffister, I. Amy Shieh, Rachell L. Vimont, Timothy J. Williams, John E. McNeal, Thomas A Stamey and David E. Clarke. RS-17053 (n-[2-(2-Cyclopropylmethoxyphenoxy)ethyl]-56-chloro-(dimethyl-1H-indole-3-ethanamine hydrochloride), a selective α_1A -adrenoceptor antagonist, displays low affinity for functional α_1 -adrenoceptors in human prostate: implications for adrenoceptor classification. *Molecular Pharmacology* 49: 209-215, 1996.

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D.R. Blue, D.W. Bonhaus, A.P.D. W. Ford, J.R. Pfister, N.A. Sharif, I.A. Shieh, R.L. vimont, T.J. Williams and D.E. Clarke. Functional evidence equating the pharmacologically defined alpha_{1A} - and cloned alpha_{1C} adrenoceptors: studies in the isolated perfused kidney of rat. *British Journal of Pharmacology* 114: 1995.

Douglas W. Bonhaus, Chinh Bach, Andrea DeSouza, F.H. Rick Salazar, Barbara D. Matsuoka, Patricia Zuppan, Hardy W. Chan and Richard M. Eglen. The pharmacology and distribution of human 5-hydroxytryptamine_{2B} (5-HT_{2B}) receptor gene products: comparison with 5-HT_{2A} and 5-HT_{2C} receptors. *British Journal of Pharmacology* 115: 622-628, 1995.

R.D. Clark, J.M. Muchowski, K.K. Weinhardt, M.P. Dillon, C-H. Lee, K.R. Bley, D.W. Bonhaus, E.H.F. Wong and R.M. Eglen. N-(quinuclidin-3-yl)-2-(1-methyl-1H-indol-3-yl)-2-oxoacetamide: a high affinity 5-HT₃ receptor agonist. *Bio-organic & Medicinal Chemistry Letters* 5: 1853-1856, 1995.

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S.S. Hegde, D.W. Bonhaus, W. Stanley, R.M. Eglen, T.M. Moy, M. Loeb, S.G. shetty, A. DesSouza and J. Krstenansky. Pharmacological evaluation of 1229U91, a novel high-affinity and selective neuropeptide Y-Y₁ antagonist. *Journal of Pharmacology and Experimental Therapeutics* 275: 1261-1266, 1995.

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L.B. Jakeman, Z.P. To, R.M. Eglén, E.H.F. Wong and D.W. Bonhaus. Quantitative Autoradiography of 5-HT₄ receptors in brains of three species using two structurally distinct radioligands, [³H]GR1138080 and [³H]BIMU-1. *Neuropharmacology* 33: 1027-1038, 1994.

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J.E. Kraus, G-C. Yeh, D.W. Bonhaus, J.V. Nadler and J.O. McNamara. Kindling induces the long-lasting expression of a novel population of NMDA receptors in hippocampal region CA3. *Journal of Neuroscience* 72: 466-472, 1994.

R.D. Clark, A. Jahangir, J.A. Langston, K.K. Weinhardt, A.B. Miller, E. Leung, D.W. Bonhaus, E.H.F. Wong and R.M. Eglén. Synthesis and preliminary pharmacological evaluation of 2-benzyloxy substituted aryl ketones as 5-HT₄ receptor antagonists. *Bio-organic & Medicinal Chemistry Letters* 4: 2481-2484, 1994.

Ann-ping Tsou, Alan Kosaka, Chinh Bach, Patti Zuppan, Calvin Yee, Leonard Tom, Scott Ramsey, Robert Alvarez, Eric Stefanich, Douglas W. Bonhaus, Lyn Jakeman, Richard M. Eglén and Hardy W. Chan. Cloning and Expression of a 5-hydroxy tryptamine₇ receptor positively coupled to adenylate cyclase. *Journal of Neurochemistry* 63: 456-464, 1994.

Erik H.F. Wong, Douglas W. Bonhaus, Irene Wu, Eric Stefanich and Richard M. Eglén: Labelling of 5-HT₃ receptors with a novel 5-HT₃ receptor ligand, [³H]RS-42358-197. *Journal of Neurochemistry* 60:921-930, 1993.

Robin D. Clark, Aaron B. Miller, Jacob Berger, David B. Repke, Klaus, K. Weinhardt,

Bruce A. Kowalczyk, Richard M. Eglen, Douglas W. Bonhaus, Chi-Ho Lee, Anton D. Michel, William L. Smith and Erik H.F. Wong: 2-(Quinuclidin-3-yl)-pyrido[4,3-b] indol-1-ones and isoquinolin-1-ones: potent conformationally restricted 5-HT₃-receptor antagonists. *Journal of Medicinal Chemistry*. 36:2645-2657, 1993.

Erik H.F. Wong, Douglas W. Bonhaus, Jeffry A. Lee, Irene Wu, Dana N. Loury and Richard M. Eglen. Different Densities of 5-HT₃ Receptors are labeled by [3H]Quipazine, [3H]GR 65630 and [3H]Granisetron. *Neuropharmacology* 32: 869-875, 1993.

R.M. Eglen, D.W. Bonhaus, K. Bley, R. Clark, S. Hedge, L.G. Johnson, E. Leung and E.H.F. Wong. RS-23597-190: A potent and selective 5-HT₄ receptor antagonist. *British Journal of Pharmacology* 110: 119-126, 1993.

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rats. Brain Research 532, 82-86, 1990.

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ABSTRACTS: - upon request

EXHIBIT

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(19) **United States**(12) **Patent Application Publication** (10) **Pub. No.: US 2004/0213816 A1**
Weiner et al. (43) **Pub. Date: Oct. 28, 2004**(54) **SELECTIVE SEROTONIN 2A/2C RECEPTOR
INVERSE AGONISTS AS THERAPEUTICS
FOR NEURODEGENERATIVE DISEASES**(76) Inventors: **David M. Weiner**, San Diego, CA
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(52) **U.S. Cl.** **424/239.1**; 514/317; 514/221;
514/567(57) **ABSTRACT**

Behavioral pharmacological data with the compound of formula (I), a novel and selective 5HT_{2A/2C} receptor inverse agonist, demonstrate in vivo efficacy in models of psychosis and dyskinesias. This includes activity in reversing MK-801 induced locomotor behaviors, suggesting that this compound may be an efficacious anti-psychotic, and activity in an MPTP primate model of dyskinesias, suggesting efficacy as an anti-dyskinesia agent. These data support the hypothesis that 5HT_{2A/2C} receptor inverse agonism may confer antipsychotic and anti-dyskinetic efficacy in humans, and indicate a use of the compound of formula (I) and related agents as novel therapeutics for Parkinson's Disease, related human neurodegenerative diseases, and psychosis.

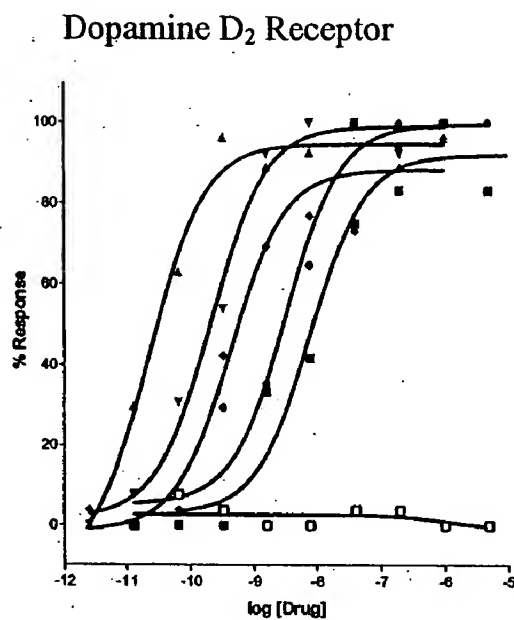


Figure 1A

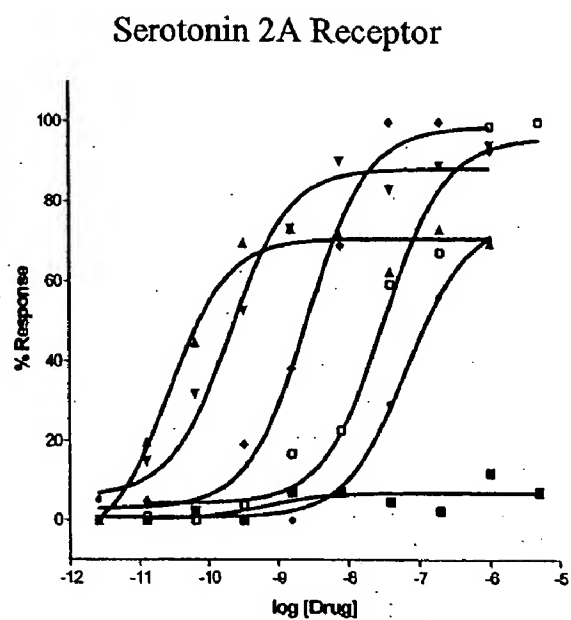


Figure 1B

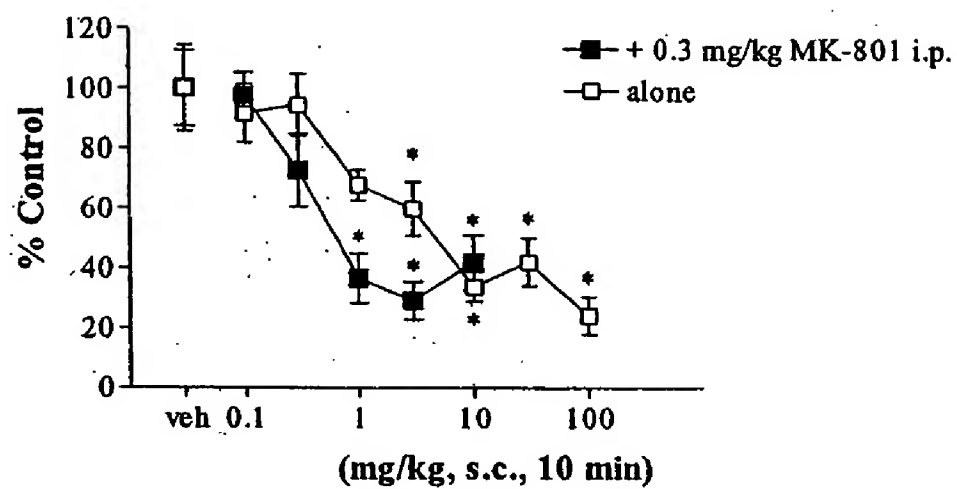


Figure 2A

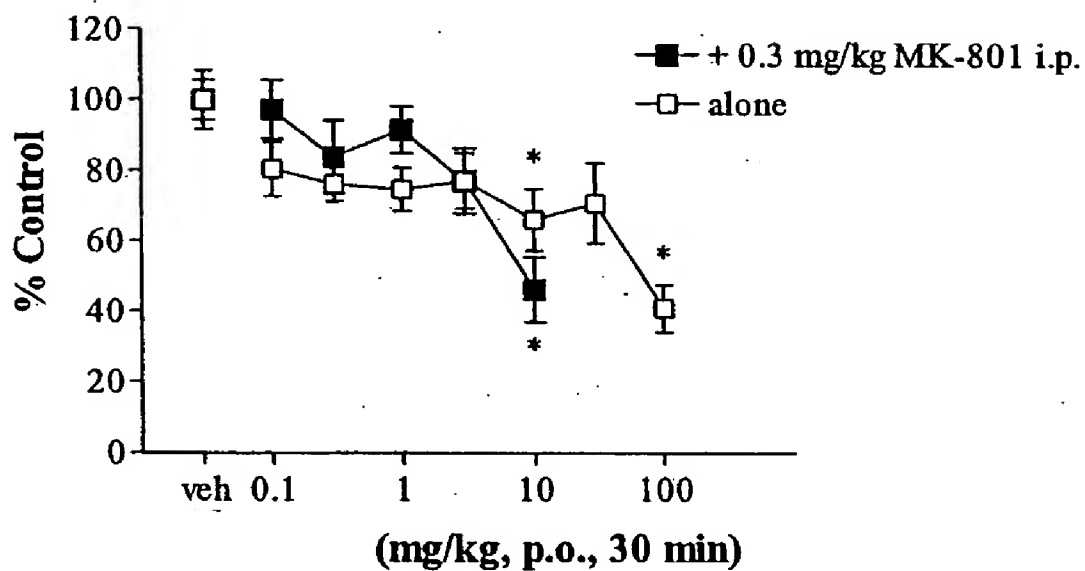


Figure 2B

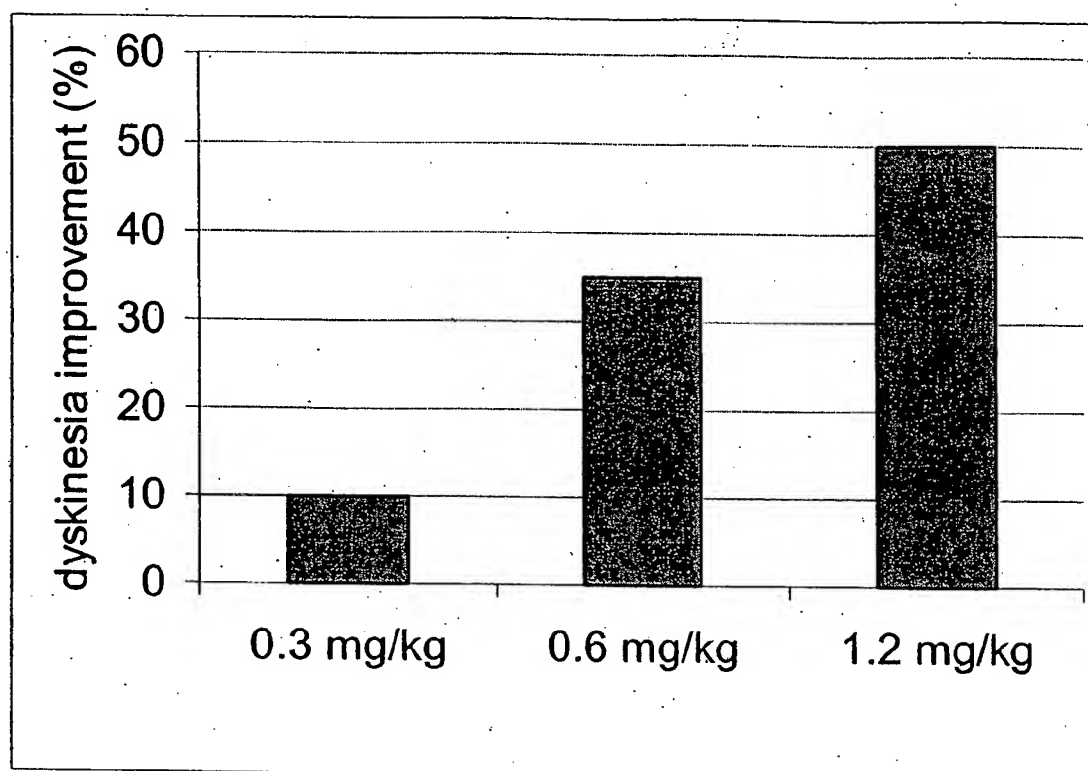


Figure 3

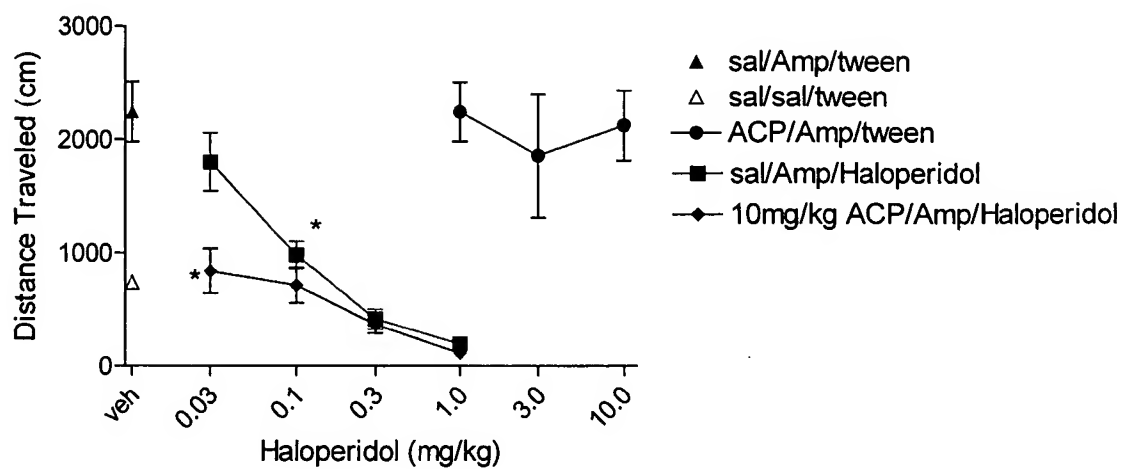


Figure 4

SELECTIVE SEROTONIN 2A/2C RECEPTOR INVERSE AGONISTS AS THERAPEUTICS FOR NEURODEGENERATIVE DISEASES

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 60/441,406, filed Jan. 16, 2003, and U.S. Provisional Application No. 60/479,346, filed Jun. 17, 2003, both by Weiner et al. and entitled "SELECTIVE SEROTONIN 2A/2C RECEPTOR INVERSE AGONISTS AS THERAPEUTICS FOR NEURODEGENERATIVE DISEASES," both of which are hereby incorporated by reference in their entireties.

FIELD OF THE INVENTION

[0002] The present invention relates to the therapeutic use of N-(1-methylpiperidin-4-yl)-N'-(4-fluorophenylmethyl)-N'-(4-(2-methylpropyloxy)phenylmethyl)carbamide and related serotonin 2A/2C receptor inverse agonists to treat a variety of human neurodegenerative diseases including Parkinson's Disease, Huntington's Disease, Lewy Body Dementia, and Alzheimer's Disease. Specifically, these agents improve motor function in Parkinson's Disease, and Huntington's Disease. Specifically, N-(1-methylpiperidin-4-yl)-N-(4-fluorophenylmethyl)-N'-(4-(2-methylpropyloxy)phenylmethyl)carbamide and related compounds can be used to control the behavioral and neuropsychiatric manifestations present in all of these disease states. Pharmaceutical compositions comprised of a combination of N-(1-methylpiperidin-4-yl)-N-(4-fluorophenylmethyl)-N'-(4-(2-methylpropyloxy)phenylmethyl)carbamide and existing therapeutic agents are also disclosed.

BACKGROUND OF THE INVENTION

[0003] Neurodegenerative disorders (NDs) are a group of related human maladies that share a common pathophysiological feature, the progressive degeneration of selective neuronal populations over the course of time. These neurodegenerative diseases include but are not limited to Alzheimer's Disease and related dementias, Parkinson's Disease, Huntington's Disease, Lewy Body Disease and related movement disorders, and Friedrich's Ataxia and related Spinocerebellar Ataxia's. Each of these disorders has unique clinical aspects including age of onset, time course of progression, neurological signs and symptoms, neuropsychiatric symptoms, and sensitivity to known therapeutic agents. In addition, the pathophysiological basis of each of these disorders is caused by genetic mechanisms unique to each disease.

[0004] Despite significant progress in elucidating the genetic causes underlying these disparate disorders, relatively little is known about the biochemical mechanisms that cause the selective neuronal degeneration common to all of them. In addition, for the most common of these disorders, including Parkinson's Disease and Alzheimer's Disease, the genetic factors that cause the rare familial forms of these diseases have been discovered, but the pathophysiological basis of the vast majority of sporadic cases is still unknown. Because of this, no specific therapeutic agents currently exist that can directly modify disease progression. Instead, clinicians utilize a variety of existing agents to provide symptomatic relief of the motor, cognitive, and neuropsychiatric

manifestations that characterize these disorders. None of these existing agents were designed and developed to specifically treat patients with NDs.

[0005] Of the various neurological symptoms that characterize the NDs, abnormalities of motor function, including bradykinesias, dyskinesias and chorea, and the emergence of neuropsychiatric symptoms, including psychosis, and affective symptoms such as anxiety and depression, are common and severely impact upon the patient's functional status and quality of life. Unfortunately, most existing therapeutic agents, including antipsychotics and antidepressants, often demonstrate efficacy, yet are very poorly tolerated in these patients. In addition, the available therapeutic agents for Parkinson's Disease, including L-dopa and dopamine agonists, while generally effective, cause the emergence of severe treatment-limiting side effects that are currently intractable to pharmacotherapy.

[0006] Multiple factors, both disease and drug related, are primarily responsible for the limited tolerability of these agents. First, patients with neurodegenerative disease are particularly sensitive to most therapeutic agents that are designed to cross the blood-brain barrier and interact with neuronal targets that confer efficacy against adverse motoric or neuropsychiatric symptoms. For instance, atypical antipsychotics are generally well tolerated by healthy volunteers, or in patients with primary psychiatric disorders like schizophrenia; brain states that are not characterized by neuronal degeneration. In contrast, when these agents are administered to patients with Parkinson's or Huntington's Disease, they display severe, treatment-limiting adverse effects on motor function, cause severe sedation, and can worsen cognitive functioning. The direct effects of the neuronal loss characteristic of NDs, and the adaptive changes that occur secondarily to this are both posited to create a neurochemical and/or neurophysiological state in ND patients that confer this extra sensitivity.

[0007] Second, the known mechanisms of action of these drugs, including antagonism of dopamine receptors, is not tolerated in some patient populations secondary to specific alterations in distinct neuronal systems. For instance, Parkinson's patients have a relatively selective degeneration of the ascending dopaminergic neuronal systems, and as a consequence of this they are deficient in central dopamine neurotransmission. It is therefore not surprising that drugs that further attenuate dopaminergic neurotransmission, by blocking dopamine receptors, are not well tolerated.

[0008] Lastly, nearly all presently known therapeutic agents lack specificity in their mechanisms of action. Antipsychotic and antidepressant drugs possess a multitude of pharmacologically relevant interactions with critical neuronal proteins including a host of cell surface receptors, ion channels, and re-uptake transporters. This lack of drug target specificity is known to confer a variety of adverse effects in non-ND patient populations, which are qualitatively and quantitatively worse in ND patients.

[0009] These observations highlight the need to develop novel therapeutic agents that are specifically designed to not only demonstrate efficacy against these particular disabling symptoms but to also possess tolerability in these specific patient populations. This can be achieved by improving the selectivity of the drug target interactions of new therapeutic agents. Specifically, the development of agents with novel

mechanisms of action that avoid the known pitfalls associated with existing agents is desired. In addition, improved selectivity can avoid the known adverse effects associated with interactions with non-efficacy conferring drug targets.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] FIG. 1 shows plots of D₂ and 5-HT_{2A} receptor agonist activity of Parkinson's Disease therapeutics as determined by the physiologically predictive, cell-based, in vivo R-SAT assay. FIG. 1A plots drug activity at human D₂ receptors. FIG. 1B plots drug activity at human Serotonin 2A receptors.

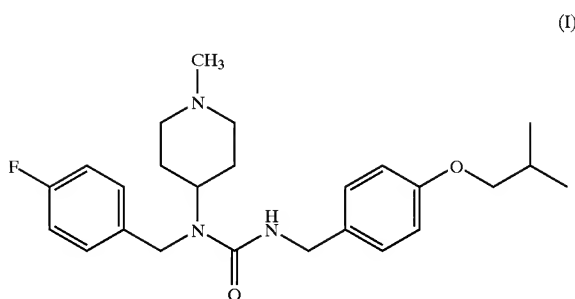
[0011] FIG. 2A is a plot of the efficacy of the compound of formula (I) in reducing MK-801 induced locomotor behaviors in rats against a control after s.c. administration over a ten (10) minute time period. FIG. 2B is a plot of the efficacy of the compound of formula (I) in reducing MK-801 induced locomotor behaviors in rats against a control after oral administration over a thirty (30) minute time period.

[0012] FIG. 3 shows a bar graph that indicates three dosage levels of the compound of formula (I) and the effect of each dosage on reducing dyskinesia in a primate model.

[0013] FIG. 4 shows the affect of the compound of formula (I) on amphetamine induced hyperactivity in mice when used in combination with varying doses of Haloperidol.

SUMMARY OF THE INVENTION

[0014] Disclosed herein is a composition comprising a compound of Formula (I):



[0015] and a pharmaceutically acceptable carrier. In some embodiments, the composition further comprises an additional therapeutic agent. In some embodiments the additional therapeutic agent is selected from levodopa (SINEMETTM, SINEMET-CRTM, bromocriptine (PARLODELTM), pergolide (PERMAXTM), ephedrine sulfate (EPHEDRINETM), pemoline CYLERTTM), mazindol (SANDOREXTM), d,1- α -methylphenethylamine (ADDERALLTM), methylphenydate (RITALINTM), pramipexole (MIRAPEXTM), modafinil (PROVIGILTM), and ropinirole (REQUIPTM). In other embodiments, the additional therapeutic agent is an anti-dyskinesia agent selected from baclofen (LioresalTM), botulinum toxin (BotoxTM), clonazepam (KlonopinTM), and diazepam (ValiumTM). In other embodiments, the additional therapeutic agent is an anti-dystonia, anti-myoclonus, or anti-tremor agent selected from baclofen (LIORESALTM), botulinum toxin (BOTOXTM),

clonazepam (KLONOPINTM), and diazepam (VALIUMTM). In other embodiments, the additional therapeutic agent is an anti-psychotic agent with dopaminergic receptor antagonism. In other embodiments, the additional therapeutic agent is an anti-psychotic agent selected from chlorpromazine (THORAZINETM), haloperidol (HALDOLTM), molindone (MOBANTM), thioridazine (MELLARILTM), a phenothiazine, a butyrophenone, diphenylbutylpiperidine (pimozide), thioxanthines (flupenthixol), substituted benzamides (sulpiride), sertindole, amisulpride, risperidone, clozapine, olanzapine, ziprasidone, aripiprazole, and their active metabolites (N-desmethyloclapine, N-desmethyloclanzapine, 9-OH-risperdone)).

[0016] Also disclosed herein is a method for treating a neurodegenerative disease comprising: identifying a patient suffering from a neurodegenerative disease and administering to the patient an effective amount of an inverse agonist selective for a serotonin receptor; whereby the dopaminergic therapy associated dyskinesia is reduced. In some embodiments, the neurodegenerative disease is Parkinson's disease, Huntington's disease, Alzheimer's disease, Spinocerebellar Atrophy, Tourette's Syndrome, Friedrich's Ataxia, Machado-Joseph's disease, Lewy Body Dementia, Dystonia, Progressive Supranuclear Palsy, or Frontotemporal Dementia. In one embodiment, the serotonin receptor is a 5HT_{2A} receptor. In another embodiment, the serotonin receptor is a 5HT_{2C} receptor. In some embodiments, the inverse agonist binds to a 5HT_{2A} receptor or a 5HT_{2C} receptor. In some embodiments, the inverse agonist is the compound of formula (I). One embodiment further comprises administering a dopaminergic agent in combination with the compound of formula (I). In some embodiments, the reagent increases dopaminergic activity and is selected from the group consisting of levodopa, SINAMETTM, SINAMETCRTM, bromocriptine (PARLODELTM), pergolide (PERMAXTM), ephedrine sulfate (EPHEDRINETM), pemoline CYLERTTM), mazindol (SANDOREXTM), d,1- α -methylphenethylamine (ADDERALLTM), methylphenydate (RITALINTM), pramipexole (MIRAPEXTM), modafinil (PROVIGILTM), and ropinirole (REQUIPTM).

[0017] Also disclosed herein is, a method for treating dyskinesia associated with dopaminergic therapy comprising: identifying a patient suffering from dopaminergic therapy associated dyskinesia and administering to the patient an effective amount of an inverse agonist selective for a serotonin receptor; whereby the dopaminergic therapy associated dyskinesia is reduced. In one embodiment the serotonin receptor is a 5HT_{2A} receptor. In another embodiment the serotonin receptor is a 5HT_{2C} receptor. In some embodiments, the inverse agonist binds to a 5HT_{2A} receptor and a 5HT_{2C} receptor. In one embodiment, the inverse agonist is the compound of formula (I). Some embodiments further comprise administering an anti-dyskinesia agent in combination with the compound of formula (I). In some embodiments, the anti-dyskinesia agent is selected from the group consisting of baclofen (LioresalTM), botulinum toxin (BotoxTM), clonazepam (KlonopinTM), and diazepam (ValiumTM). In some embodiments, the patient suffers from a neurodegenerative disease selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, Spinocerebellar Atrophy, Tourette's Syndrome, Friedrich's Ataxia, Machado-Joseph's disease, Lewy

Body Dementia, Dystonia, Progressive Supranuclear Palsy, and Frontotemporal Dementia.

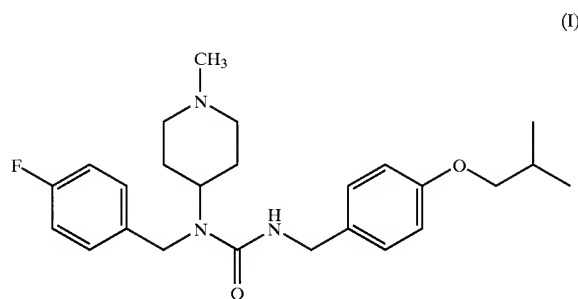
[0018] Further disclosed herein is a method for treating dystonia, myoclonus, or tremor associated with dopaminergic therapy comprising: identifying a patient suffering from dopaminergic therapy associated dystonia, myoclonus, or tremor; and administering to the patient an effective amount of an inverse agonist selective for a serotonin receptor; whereby the dopaminergic therapy associated dystonia, myoclonus, or tremor is reduced. In one embodiment the serotonin receptor is a 5HT_{2A} receptor. In another embodiment, the serotonin receptor is a 5HT_{2C} receptor. In some embodiments, the inverse agonist binds to a 5HT_{2A} receptor and a 5HT_{2C} receptor. In some embodiments, the inverse agonist is the compound of formula (I). Some embodiments further comprise an anti-dystonia, anti-myoclonus, or anti-tremor agent in combination with the compound of formula (I). In some embodiments, the anti-dystonia, anti-myoclonus, or anti-tremor agent is selected from the group consisting of baclofen (LIORESALTM), botulinum toxin (BOTOXTM), clonazepam (KLONOPINTM), and diazepam (VALIUMTM).

[0019] Also disclosed herein is a method for treating psychosis associated with dopaminergic therapy comprising: identifying a patient suffering from dopaminergic therapy associated psychosis; and administering to the patient an effective amount of an inverse agonist selective for a serotonin receptor; whereby symptoms of dopaminergic therapy associated psychosis is reduced. In one embodiment the serotonin receptor is a 5HT_{2A} receptor. In another embodiment the serotonin receptor is a 5HT_{2C} receptor. In some embodiments the inverse agonist binds to a 5HT_{2A} receptor and a 5HT_{2C} receptor. In some embodiments the inverse agonist is the compound of formula (I). Some embodiments further comprise an anti-psychotic agent in combination with the compound of formula (I). In some embodiments, the anti-psychotic agent is selected from the group consisting of chlorpromazine (THORAZINETM), haloperidol (HALDOLTM), molindone (MOBANTM), thioridazine (MELLARILTM), a phenothiazine, a butyrophenone, diphenylbutylpiperidine (pimozide), thioxanthines (flupenthixol), substituted benzamides (sulpiride), sertindole, amisulpride, risperidone, clozapine, olanzapine, ziprasidone, aripiprazole, and their active metabolites (N-desmethyloclozapine, N-desmethyloanzapine, 9-OH-risperdone)). In some embodiments, the patient suffers from a neurodegenerative disease selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, Spinocerebellar Atrophy, Tourette's Syndrome, Friedrich's Ataxia, Machado-Joseph's disease, Lewy Body Dementia, Dystonia, Progressive Supranuclear Palsy, and Frontotemporal Dementia.

[0020] Also disclosed herein is a method for treating a neuropsychiatric disease comprising: identifying a patient suffering from a neuropsychiatric disease; and administering to the patient an effective amount of an inverse agonist selective for a serotonin receptor. In some embodiments, the neuropsychiatric disease is selected from the group consisting of schizophrenia, schizoaffective disorders, mania, behavioral disturbances associated with dementia and psychotic depression. In some embodiments the serotonin receptor is a 5HT_{2A} receptor. In some embodiments the serotonin receptor is a 5HT_{2C} receptor. In some embodi-

ments the inverse agonist binds to a 5HT_{2A} receptor or a 5HT_{2C} receptor. In one embodiment, the inverse agonist is the compound of formula (I). Some embodiments further comprise administering an antipsychotic agent in combination with the inverse agonist, the anti-psychotic agent selected from the group consisting of chlorpromazine (THORAZINETM), haloperidol (HALDOLTM), molindone (MOBANTM), thioridazine (MELLARILTM), a phenothiazine, a butyrophenone, diphenylbutylpiperidine (pimozide), thioxanthines (flupenthixol), substituted benzamides (sulpiride), sertindole, amisulpride, risperidone, clozapine, olanzapine, ziprasidone, aripiprazole, and their active metabolites (N-desmethyloclozapine, N-desmethyloanzapine, 9-OH-risperdone)).

[0021] Also disclosed herein is a compound having the structure of Formula (I):



[0022] Additionally disclosed herein is a method of inhibiting an activity of a monoamine receptor comprising contacting the monoamine receptor or a system containing the monoamine receptor with an amount of the compound of formula (I) that is effective in inhibiting the activity of the monoamine receptor. In some embodiments, the monoamine receptor is a serotonin receptor. In one embodiment the serotonin receptor is the 5-HT_{2A} subclass. In some embodiments the serotonin receptor is in the central nervous system. In some embodiments the serotonin receptor is in the peripheral nervous system. In some embodiments the serotonin receptor is in blood cells or platelets. In some embodiments the serotonin receptor is mutated or modified. In some embodiments the activity is signaling activity. In some embodiments the activity is constitutive. In some embodiments the activity is associated with serotonin receptor activation.

[0023] Also disclosed herein is a method of inhibiting an activation of a monoamine receptor comprising contacting the monoamine receptor or a system containing the monoamine receptor with an amount of the compound of formula (I) that is effective in inhibiting the activation of the monoamine receptor. In some embodiments, the activation is by an agonistic agent. In some embodiments the agonistic agent is exogenous. In some embodiments the agonistic agent is endogenous. In some embodiments the activation is constitutive. In some embodiments the monoamine receptor is a serotonin receptor. In some embodiments the serotonin receptor is the 5-HT_{2A} subclass. In some embodiments the serotonin receptor is in the central nervous system. In some embodiments the serotonin receptor is in the peripheral nervous system. In some embodiments the serotonin recep-

tor is in blood cells or platelets. In some embodiments the serotonin receptor is mutated or modified.

[0024] Also disclosed herein is a method of treating a disease condition associated with a monoamine receptor comprising administering to a subject in need of such treatment a therapeutically effective amount of the compound of formula (I). In some embodiments the disease condition is selected from the group consisting of schizophrenia, psychosis, migraine, hypertension, thrombosis, vasospasm, ischemia, depression, anxiety, sleep disorders and appetite disorders. In some embodiments the disease condition is associated with dysfunction of a monoamine receptor. In some embodiments, the disease condition is associated with activation of a monoamine receptor. In some embodiments, the disease condition is associated with increased activity of monoamine receptor. In some embodiments, the monoamine receptor is a serotonin receptor. In some embodiments the serotonin receptor is the 5-HT_{2A} subclass. In some embodiments the serotonin receptor is in the central nervous system. In some embodiments the serotonin receptor is in the peripheral nervous system. In some embodiments the serotonin receptor is in blood cells or platelets. In some embodiments, the serotonin receptor is mutated or modified.

[0025] Also disclosed herein is a method of treating schizophrenia comprising administering to a subject in need of such treatment a therapeutically effective amount of the compound of formula (I).

[0026] Also disclosed herein is a method of treating migraine comprising administering to a subject in need of such treatment a therapeutically effective amount of the compound of formula (I).

[0027] Also disclosed herein is a method of treating psychosis comprising administering to a subject in need of such treatment a therapeutically effective amount of the compound of formula (I).

[0028] Also disclosed herein is a method for identifying a genetic polymorphism predisposing a subject to being responsive to the compound of formula (I), comprising: administering to a subject a therapeutically effective amount of said compound; measuring the response of said subject to said compound, thereby identifying a responsive subject having an ameliorated disease condition associated with a monoamine receptor; and identifying a genetic polymorphism in the responsive subject, wherein the genetic polymorphism predisposes a subject to being responsive to said compound. In some embodiments the ameliorated disease condition is associated with the 5-HT class or 5-HT_{2A} subclass of monoaminergic receptors.

[0029] Additionally disclosed herein is a method for identifying a subject suitable for treatment with the compound of claim 48, comprising detecting the presence of a polymorphism in a subject wherein the polymorphism predisposes the subject to being responsive to the compound, and wherein the presence of the polymorphism indicates that the subject is suitable for treatment with the compound of formula (I).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0030] Definitions

[0031] For the purpose of the current disclosure, the following definitions shall in their entireties be used to

define technical terms, and shall also, in their entireties, be used to define the scope of the composition of matter for which protection is sought in the claims.

[0032] "Constitutive activity" is defined as the elevated basal activity of a receptor that is independent of the presence of an agonist. Constitutive activity of a receptor may be measured using a number of different methods, including cellular (e.g., membrane) preparations (see, e.g., Barr & Manning, *J. Biol. Chem.* 272:32979-87 (1997)), purified reconstituted receptors with, or without the associated G-protein in phospholipid vesicles (Cerione et al., *Biochemistry* 23:4519-25 (1984)), and functional cellular assays (U.S. Patent Application Ser. No. 60/103,317) or any other method known in the art.

[0033] "Agonist" is defined as a compound that increases the basal activity of a receptor when it contacts the receptor.

[0034] An "antagonist" is defined as a compound that competes with an agonist or inverse agonist for binding to a receptor, thereby blocking the action of an agonist or inverse agonist on the receptor. However, an antagonist (also known as a "neutral" antagonist) has no effect on constitutive receptor activity.

[0035] An "inverse agonist" is defined as a compound that decreases the basal activity of a receptor (i.e., signaling mediated by the receptor). Such compounds are also known as negative antagonists. An inverse agonist is a ligand for a receptor that causes the receptor to adopt an inactive state relative to a basal state occurring in the absence of any ligand. Thus, while an antagonist can inhibit the activity of an agonist, an inverse agonist is a ligand that can alter the conformation of the receptor in the absence of an agonist. The concept of an inverse agonist has been explored by Bond et al. in *Nature* 374:272 (1995). More specifically, Bond et al. have proposed that unliganded β_2 -adrenoceptor exists in an equilibrium between an inactive conformation and a spontaneously active conformation. Agonists are proposed to stabilize the receptor in an active conformation. Conversely, inverse agonists are believed to stabilize an inactive receptor conformation. Thus, while an antagonist manifests its activity by virtue of inhibiting an agonist, an inverse agonist can additionally manifest its activity in the absence of an agonist by inhibiting the spontaneous conversion of an unliganded receptor to an active conformation.

[0036] The "5-HT_{2A} receptor" is defined as a receptor, having an activity corresponding to the activity of the human serotonin receptor subtype, which was characterized through molecular cloning and pharmacology as detailed in Saltzman et al., *Biochem. Biophys. Res. Comm.* 181:1469-78; and Julius et al., *Proc. Natl. Acad. Sci. USA* 87:928-932, the disclosures of which are incorporated herein by reference in their entireties.

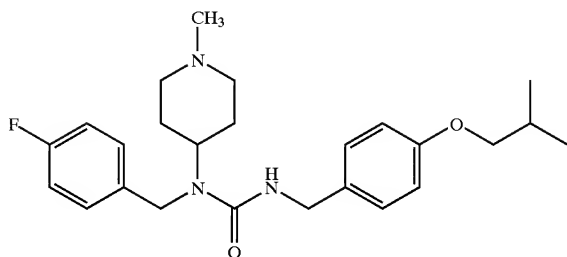
[0037] The term "subject" refers to an animal, preferably a mammal, most preferably a human, who is the object of treatment, observation or experiment.

[0038] "Selective" is defined as a property of a compound whereby an amount of the compound sufficient to effect a desired response from a particular receptor type, subtype, class or subclass with significantly less or substantially little or no effect upon the activity other receptor types. For example, a selective compound may have at least a 10-fold greater effect on activity of the desired receptor than on other

receptor types. In some cases, a selective compound may have at least a 20-fold greater effect on activity of the desired receptor than on other receptor types, or at least a 50-fold greater effect, or at least a 100-fold greater effect, or at least a 1000-fold greater effect, or at least a 10,000-fold greater effect, or at least a 100,000-fold greater effect, or more than a 100,000-fold greater effect. "Selectivity" or "selective," as an inverse agonist is understood as a property of the compound of the invention whereby an amount of compound that effectively inversely agonizes the 5-HT_{2A} receptor, and thereby decreases its activity, causes little or no inverse agonistic or antagonistic activity at other, related or unrelated, receptors. In particular, in one embodiment, a compound has surprisingly been found not to interact strongly with other serotonin receptors (5-HT 1A, 1B, 1D, 1E, 1F, 2B, 2C, 4A, 6, and 7) at concentrations where the signaling of the 5-HT_{2A} receptor is strongly or completely inhibited. In one embodiment, the compound is also selective with respect to other monoamine-binding receptors, such as the dopaminergic, histaminergic, adrenergic and muscarinic receptors. Compounds that are highly selective for 5-HT_{2A} receptors may have a beneficial effect in the treatment of psychosis, schizophrenia or similar neuropsychiatric disorders, while avoiding adverse effects associated with drugs hitherto suggested for this purpose.

[0039] Some embodiments described herein relate to serotonin 2A or 2C receptor inverse agonists, including compositions and methods for treating certain side-effects caused or exacerbated by dopaminergic agent-associated therapies commonly used in treating neurodegenerative diseases. For example, the compounds disclosed herein have utility in reducing dyskinesia and psychosis associated with dopaminergic therapies used in treating Parkinson's disease, a neurodegenerative disease. According to one embodiment, the compound N-(1-methylpiperidin-4-yl)-N-(4-fluorophenylmethyl)-N'-(4-(2-methylpropyloxy)phenylmethyl)-carbamide having the structure of formula (I) is provided:

(I)



[0040] One embodiment relates to a composition comprising the compound of formula (I) and a pharmaceutically acceptable carrier. The composition may also contain other compounds such as compounds for treating dyskinesia, dystonia, or psychosis.

[0041] According to one embodiment, the tartrate salt of the compound, N-(1-methylpiperidin-4-yl)-N-(4-fluorophenylmethyl)-N'-(4-(2-methylpropyloxy)phenylmethyl)-carbamide is a potent, selective, orally bioavailable 5-HT_{2A} receptor inverse agonist. The compound of formula (I) also possesses lesser potency as a 5-HT_{2C} receptor

inverse agonist and lacks intrinsic activity at the remaining monoaminergic receptor subtypes. Perhaps most notably, the compound of formula (I) lacks activity at dopamine receptor subtypes. (See U.S. patent application Ser. No. 09/800,096, which is hereby incorporated by reference in its entirety). Extensive behavioral pharmacological profiling of the compound of formula (I), including pre-clinical models of antipsychotic and anti-dyskinetic drug actions, support the therapeutic use of the compound in Parkinson's Disease and related human neurodegenerative diseases.

[0042] Parkinson's Disease (PD) is a common and progressive neurodegenerative disease. Current estimates suggest that nearly 900,000 individuals in the United States have PD and that the prevalence is increasing as the US population ages. Dopamine receptor agonists are used to alleviate the symptoms of PD, such as motoric dysfunction. Unfortunately, the protracted use of these dopaminergic agents causes, over time, neuropsychiatric (psychosis) and troublesome motor (dyskinesia) side effects in 30 to 80% of patients, respectively.

[0043] Antipsychotics and dopamine receptor antagonists can be effective in ameliorating these adverse effects. Unfortunately, many of these compounds significantly worsen motor function in PD patients secondary to their hypodopaminergic state. Biochemical and pharmacological data support the hypothesis that potentiation of serotonergic neurotransmission may be pathophysiologically related to the development of dyskinesias and psychosis in these patients. While not being bound by this theory, the compounds disclosed herein were selected to exploit the relationship of serotonergic activity and the negative side-effects associated with dopaminergic therapy.

[0044] L-dopa is a typical dopaminergic compound used to treat PD. L-dopa has been shown to increase central serotonin release, turnover, and metabolite concentrations in rodent brain. Direct acting dopamine receptor agonists like pergolide possess, in addition to their dopamine receptor agonist properties, potent agonist activity at serotonin 2A (5-HT_{2A}) and 2C (5-HT_{2C}) receptors as demonstrated by various in vitro pharmacological assays.

[0045] In one embodiment, the compounds disclosed herein can be used to treat many side-effects that arise from dopaminergic therapy. For example, the disclosed compounds are also useful for treatment of dyskinesia or psychosis caused or exacerbated as a side-effect of other therapeutic agents such as L-dopa. In one embodiment, the compounds are preferably used for the treatment of dyskinesia or psychosis associated with L-dopa treatment.

[0046] The compounds may be used to treat existing dyskinesia or psychosis or may be used prophylactic fashion when for example, it is considered necessary to initiate L-dopa therapy and it is feared that dyskinesia or psychosis may develop.

[0047] The compounds may be used to treat dyskinesia or psychosis as a monotherapy or as an adjunct to medications to prevent or treat dyskinesia or psychosis side-effects caused by the medicament or alternatively the compounds may be given in combination with other compounds which also reduce dyskinesia.

[0048] In some embodiments, the compounds described herein can be formulated into compositions for administra-

tion to patients in need thereof. Appropriate compositions can take a number of different forms depending on how the composition is to be used. For example, the composition may be in the form of a powder, tablet, capsule, liquid, ointment, cream, gel, hydrogel, aerosol, spray, micelle, liposome or any other pharmaceutically acceptable form. One of ordinary skill in the art would readily appreciate that an appropriate vehicle for use with the disclosed compounds of the invention should be one that is well tolerated by a recipient of the composition. The vehicle should also readily enable the delivery of the compounds to appropriate target receptors. For example, one of ordinary skill in the art would know to consult *Pharmaceutical Dosage Forms and Drug Delivery Systems*, by Ansel, et al., Lippincott Williams & Wilkins Publishers; 7th ed. (1999) or a similar text for guidance regarding such formulations.

[0049] The composition of the invention may be used in a number of ways. For instance, systemic administration may be required in which case the disclosed compounds can be formulated into a composition that can be ingested orally in the form of a tablet, capsule or liquid. Alternatively the composition may be administered by injection into the blood stream. Injections may be intravenous (bolus or infusion) or subcutaneous (bolus or infusion). The disclosed compounds can also be administered centrally by means of intracerebral, intracerebroventricular, or intrathecal delivery.

[0050] The compound may also be used with a time delayed release device. Such devices may, for example, be inserted under the skin and the compound may be released over weeks or months. Such a device may be particularly useful for patients with long term dyskinesia such as patients on continuous L-dopa therapy for the treatment of PD. The devices may be particularly advantageous when a compound is used which would normally require frequent administration (e.g., frequent injection).

[0051] It will be readily appreciated that the amount of a compound required is determined by biological activity and bioavailability which in turn depends on the mode of administration, the physicochemical properties of the compound employed and whether the compound is being used as a monotherapy or in a combined therapy. The frequency of administration will also be influenced by the above mentioned factors and particularly the half-life of the compound within the subject being treated.

[0052] One of ordinary skill in the art would appreciate that specific formulations of compositions and precise therapeutic regimes (such as daily doses of the compounds and the frequency of administration) can be determined using known procedures. Such procedures conventionally employed by the pharmaceutical industry include in vivo experimentation and clinical trials.

[0053] Generally, a daily dose of between 0.01 $\mu\text{g/kg}$ of body weight and 1.0 g/kg of body weight of a serotonin 2A/2C receptor inverse agonist can be used with the methods disclosed herein. In one embodiment, the daily dose is between 0.01 mg/kg of body weight and 100 mg/kg of body weight, or any milligram or half-milligram quantity in this disclosed range, e.g., 1.5, 2, 2.5, etc.

[0054] Daily doses may be given as a single administration (e.g. a daily tablet for oral consumption or as a single daily injection). Alternatively the compound used may

require administration twice or more times during a day, depending on the kinetics of the drug associated with the individual patient. Alternatively a slow release device may be used to provide optimal doses to a patient without the need to administer repeated doses.

[0055] Biochemical Evidence

[0056] The cornerstone of current pharmacological intervention in PD remains L-dopa based therapies. L-dopa readily crosses the blood brain barrier, is taken up by neurons and undergoes rapid enzymatic conversion to dopamine, via L-aromatic acid decarboxylase (LAAD) activity in dopaminergic neurons. The increased availability and release of dopamine from these neurons clearly leads to increased dopaminergic transmission, and clinical efficacy in reversing the motoric effects of the hypo-dopaminergic state observed in PD. However, L-dopa lacks specificity for dopaminergic systems, and LAAD is widely expressed in brain. Early biochemical observations in rat brain noted that L-dopa substantially reduced central serotonergic stores, and increased the concentration of the principle serotonin metabolite of 5-hydroxyindoleacetic acid (5-HIAA) (1). Histochemical approaches have demonstrated that L-dopa accumulates in serotonergic neurons, and neurotransmitter release experiments have demonstrated that L-dopa markedly increased the release of both dopamine and serotonin, that release of serotonin is dependent upon LAAD activity, and that it is not eliminated by the selective destruction of dopaminergic neurons (2,3). These observations suggest that the administration of L-dopa to PD patients results in marked increases in the release of central serotonin, potentiating serotonergic neurotransmission. Finally, post-mortem biochemical analysis of PD patients that developed psychosis, when compared to a matched group that did not develop neuropsychiatric disturbances, found that the patients with psychosis had significant elevations in serotonin and 5-HIAA levels in multiple cortical and sub-cortical structures, most notably various mesencephalic nuclei including the red nucleus (4).

[0057] Serotonin or 5-hydroxytryptamine (5-HT) plays a significant role in the functioning of the mammalian body. In the central nervous system, 5-HT is an important neurotransmitter and neuromodulator that is implicated in such diverse behaviors and responses as sleeping, eating, locomotion, perceiving pain, learning and memory, sexual behavior, controlling body temperature and blood pressure. In the spinal column, serotonin plays an important role in the control systems of the afferent peripheral nociceptors (Moulinier, *Rev. Neurol.* 150:3-15, (1994)). Peripheral functions in the cardiovascular, hematological, and gastrointestinal systems have also been ascribed to 5-HT. 5-HT has been found to mediate a variety of contractile, secretory, and electrophysiologic effects including vascular and nonvascular smooth muscle contraction, and platelet aggregation. (Fuller, *Biology of Serotonergic Transmission*, 1982; Bottilin, *Serotonin In Mental Abnormalities* 1:316 (1978); Barchas, et al., *Serotonin and Behavior*, (1973)). The 5-HT_{2A} receptor subtype (also referred to as subclass) is widely yet discretely expressed in the human brain, including many cortical, limbic, and forebrain regions postulated to be involved in the modulation of higher cognitive and affective functions. This receptor subtype is also expressed on mature platelets where it mediates, in part, platelet aggregation, one of the initial steps in the process of vascular thrombosis.

[0058] Given the broad distribution of serotonin within the body, it is understandable that tremendous interest in drugs that affect serotonergic systems exists (Gershon, et al, *The Peripheral Actions of 5-Hydroxytryptamine*, 246 (1989); Saxena, et al, *J. Cardiovascular Pharmacol.* 15: Supp. 7 (1990)). Serotonin receptors are members of a large human gene family of membrane-spanning proteins that function as transducers of intercellular communication. They exist on the surface of various cell types, including neurons and platelets, where, upon their activation by either their endogenous ligand serotonin or exogenously administered drugs, they change their conformational structure and subsequently interact with downstream mediators of cellular signaling. Many of these receptors, including the 5-HT_{2A} subclass, are G-protein coupled receptors (GPCRs) that signal by activating guanine nucleotide binding proteins (G-proteins), resulting in the generation, or inhibition of, second messenger molecules such as cyclic AMP, inositol phosphates, and diacylglycerol. These second messengers then modulate the function of a variety of intracellular enzymes, including kinases and ion channels, which ultimately affect cellular excitability and function.

[0059] At least 15 genetically distinct 5-HT receptor subtypes have been identified and assigned to one of seven families (5-HT₁₋₇). Each subtype displays a unique distribution, preference for various ligands, and functional correlate(s). Serotonin may be an important component in various types of pathological conditions such as certain psychiatric disorders (depression, aggressiveness, panic attacks, obsessive compulsive disorders, psychosis, schizophrenia, suicidal tendency), certain neurodegenerative disorders (Alzheimer-type dementia, Parkinsonism, Huntington's chorea), anorexia, bulimia, disorders associated with alcoholism, cerebral vascular accidents, and migraine (Meltzer, *Neuropsychopharmacology*, 21:106S-115S (1999); Barnes & Sharp, *Neuropharmacology*, 38:1083-1152 (1999); Glennon, *Neurosci. Biobehavioral Rev.*, 14:35 (1990)). Recent evidence strongly implicates the 5-HT₂ receptor subtype in the etiology of such medical conditions as hypertension, thrombosis, migraine, vasospasm, ischemia, depression, anxiety, psychosis, schizophrenia, sleep disorders and appetite disorders.

[0060] Schizophrenia is a particularly devastating neuropsychiatric disorder that affects approximately 1% of the human population. It has been estimated that the total financial cost for the diagnosis, treatment, and lost societal productivity of individuals affected by this disease exceeds 2% of the gross national product (GNP) of the United States. Current treatment primarily involves pharmacotherapy with a class of drugs known as antipsychotics. Antipsychotics are effective in ameliorating positive symptoms (e.g., hallucinations and delusions), yet they frequently do not improve negative symptoms (e.g., social and emotional withdrawal, apathy, and poverty of speech).

[0061] Currently, nine major classes of antipsychotics are prescribed to treat psychotic symptoms. Use of these compounds is limited, however, by their side effect profiles. Nearly all of the "typical" or older generation compounds have significant adverse effects on human motor function. These "extrapyramidal" side effects, so termed due to their effects on modulatory human motor systems, can be both acute (e.g., dystonic reactions, a potentially life threatening but rare neuroleptic malignant syndrome) and chronic (e.g.,

akathisia, tremors, and tardive dyskinesia). Drug development efforts have, therefore, focused on newer "atypical" agents free of these adverse effects.

[0062] Antipsychotic drugs have been shown to interact with a large number of central monoaminergic neurotransmitter receptors, including dopaminergic, serotonergic, adrenergic, muscarinic, and histaminergic receptors. It is likely that the therapeutic and adverse effects of these drugs are mediated by distinct receptor subtypes. The high degree of genetic and pharmacological homology between these receptor subtypes has hampered the development of subtype-selective compounds, as well as the determination of the normal physiologic or pathophysiologic role of any particular receptor subtype. Thus there is a need to develop drugs that are selective for individual receptor classes and subclasses amongst monoaminergic neurotransmitter receptors.

[0063] The prevailing theory for the mechanism of action of antipsychotic drugs involves antagonism of dopamine D₂ receptors. Unfortunately, it is likely that antagonism of dopamine D₂ receptors also mediates the extrapyramidal side effects. Antagonism of 5-HT_{2A} is an alternate molecular mechanism for drugs with antipsychotic efficacy, possibly through antagonism of heightened or exaggerated signal transduction through serotonergic systems. 5-HT_{2A} antagonists are therefore good candidates for treating psychosis without extrapyramidal side effects.

[0064] Traditionally, these receptors have been assumed to exist in a quiescent state unless activated by the binding of an agonist (a drug that activates a receptor). It is now appreciated that many, if not most, of the GPCR monoamine receptors, including serotonin receptors, can exist in a partially activated state in the absence of their endogenous agonists. This increased basal activity (constitutive activity) can be inhibited by compounds called inverse agonists. Both agonists and inverse agonists possess intrinsic activity at a receptor, in that they alone can activate or inactivate these molecules, respectively. In contrast, classic or neutral antagonists compete against agonists and inverse agonists for access to the receptor, but do not possess the intrinsic ability to inhibit elevated basal or constitutive receptor-responses.

[0065] We have elucidated an important aspect of 5-HT_{2A} receptor function by applying the Receptor Selection and Amplification Technology (U.S. Pat. No. 5,707,798, 1998; *Chem. Abstr.* 128:111548 (1998) and citations therein), to the study of the 5-HT₂ subclass of serotonin receptors. R-SAT is a phenotypic assay of receptor function that involves the heterologous expression of receptors in mammalian fibroblasts. Using this technology we were able to demonstrate that native 5-HT_{2A} receptors possess significant constitutive, or agonist-independent, receptor activity (U.S. Patent Application Ser. No. 60/103,317, herein incorporated by reference). Furthermore, by directly testing a large number of centrally acting medicinal compounds with known clinical activity in neuropsychiatric disease, we determined that compounds with antipsychotic efficacy all shared a common molecular property. Nearly all of these compounds, which are used by psychiatrists to treat psychosis, were found to be potent 5-HT_{2A} inverse agonists. This unique clinico-pharmacologic correlation at a single

receptor subtype is compelling evidence that 5-HT_{2A} receptor inverse agonism is a molecular mechanism of antipsychotic efficacy in humans.

[0066] Detailed pharmacological characterization of a large number of antipsychotic compounds revealed that they possess broad activity at multiple related receptor subtypes. Most of these compounds display agonist, competitive antagonist, or inverse agonist activity at multiple monoaminergic receptor subtypes, including serotonergic, dopaminergic, adrenergic, muscarinic and histaminergic receptors. This broad activity is likely responsible for the sedating, hypotensive, and motor side effects of these compounds. It would therefore be of great advantage to develop compounds that are selective inverse agonists of the 5-HT_{2A} receptor, but which have little or no activity on other monoamine receptor subtypes, especially dopamine D₂ receptors. Such compounds may be useful in the treatment of human disease (e.g., as anti-psychotics), and may avoid the adverse side effects associated with non-selective receptor interactions.

[0067] The compound of formula (I) is active at monoamine receptors, specifically serotonin receptors. In one embodiment, the compound acts as inverse agonist at the 5-HT_{2A} receptor. Thus, experiments performed on cells transiently expressing the human phenotype of said receptor have shown that the compound of formula (I) attenuates the signaling of such receptors in the absence of additional ligands acting upon the receptor. The compound has thus been found to possess intrinsic activity at this receptor and is able to attenuate the basal, non-agonist-stimulated, constitutive signaling responses that the 5-HT_{2A} receptor displays. The observation that the compound of formula (I) is an inverse agonist also indicates that the compound has the ability to antagonize the activation of 5-HT_{2A} receptors that is mediated by endogenous agonists or exogenous synthetic agonist ligands.

[0068] In one embodiment, the compound of formula (I) shows a relatively high degree of selectivity towards the 5-HT_{2A} subtype of serotonin receptors relative to other subtypes of the serotonin (5-HT) family of receptors as well as to other receptors, most particularly the monoaminergic G-protein coupled receptors, such as dopamine receptors.

[0069] The compound of formula (I) may therefore be useful for treating or alleviating symptoms of disease conditions associated with impaired function, in particular elevated levels of activity, of especially 5-HT_{2A} receptors, whether this impaired function is associated with improper levels of receptor stimulation or phenotypical aberrations.

[0070] Others have previously hypothesized that certain neuropsychological diseases might be caused by altered levels of constitutive activity of monoamine receptors. Such constitutive activity might be modified via contacting the relevant receptor with a synthetic inverse agonist. By directly testing a large number of centrally acting medicinal compounds with known clinical activity in neuropsychiatric disease, we determined that compounds with antipsychotic efficacy all shared a common molecular property. Nearly all of these compounds that are used by psychiatrists to treat psychosis were found to be potent 5-HT_{2A} inverse agonists. This correlation is compelling evidence that 5-HT_{2A} receptor inverse agonism is a molecular mechanism of antipsychotic efficacy in humans.

[0071] Detailed pharmacological characterization of a large number of antipsychotic compounds in our laboratory revealed that they possess broad activity at multiple related receptor subtypes. Most of these compounds display either agonist, competitive antagonist, or inverse agonist activity at multiple monoaminergic receptor subtypes including serotonergic, dopaminergic, adrenergic, muscarinic and histaminergic receptors. This broad activity is likely responsible for the sedating, hypotensive, and motor side effects of these compounds. In one embodiment, the compound of formula (I) possesses efficacy as, for example, a novel antipsychotic, but will have fewer or less severe side effects than existing compounds.

[0072] In one embodiment a method is provided to inhibit activity of a monoamine receptor. This method comprises contacting a monoamine receptor or a system containing the monoamine receptor, with an effective amount of the compound of formula (I). According to one embodiment, the monoamine receptor is a serotonin receptor. In one embodiment, the compound is selective for the 5-HT_{2A} receptor subclass. In another embodiment, the compound has little or substantially no activity to other types of receptors, including other serotonergic receptors and most particularly, monoaminergic G-protein coupled receptors, such as dopaminergic receptors.

[0073] The system containing the monoamine receptor may, for example, be a subject such as a mammal, non-human primate or a human. The receptor may be located in the central or peripheral nervous system, blood cells or platelets.

[0074] The system may also be an in vivo or in vitro experimental model, such as a cell culture model system that expresses a monoamine receptor, a cell-free extract thereof that contains a monoamine receptor, or a purified receptor. Non-limiting examples of such systems are tissue culture cells expressing the receptor or extracts or lysates thereof. Cells that may be used in the present method include any cells capable of mediating signal transduction via monoamine receptors, especially the 5-HT_{2A} receptor, either via endogenous expression of this receptor (e.g., certain types of neuronal cells lines, for example, natively express the 5-HT_{2A} receptor), or following transfection of cells with plasmids containing the receptor gene. Such cells are typically mammalian cells (or other eukaryotic cells, such as insect cells or *Xenopus* oocytes), because cells of lower organisms generally lack the appropriate signal transduction pathways for the present purpose. Examples of suitable cells include: the mouse fibroblast cell line NIH 3T3 (ATCC CRL 1658), which responds to transfected 5-HT_{2A} receptors by stimulating growth; RAT 1 cells (Pace et al., *Proc. Natl. Acad. Sci. USA* 88:7031-35 (1991)); and pituitary cells (Vallar et al., *Nature* 330:556-58 (1987)). Other useful mammalian cells for the present method include HEK 293 cells, CHO cells, and COS cells.

[0075] One embodiment provides methods of inhibiting activity of a native, mutated or modified monoamine receptor. Also provided are kits for performing the same. In one embodiment, the activity of the receptor is a signaling activity. In another embodiment, the activity of the receptor is the constitutive basal activity of the receptor.

[0076] In one embodiment, the activity of the receptor is a response, such as a signaling response, to an endogenous

agonist, such as 5-HT, or an exogenous agonistic agent, such as a drug or other synthetic ligand. The compound of formula (I) may act by either inversely agonizing or antagonizing the receptor.

[0077] In one embodiment, the compound of formula (I) is an inverse agonist selective for the 5-HT_{2A} receptor and the compound has little or substantially no activity toward other serotonergic or other monoaminergic receptors, such as dopaminergic receptors.

[0078] In a further embodiment, a method is provided for inhibiting an activation of a monoamine receptor comprising contacting the monoamine receptor, or a system containing the monoamine receptor, with the compound of formula (I). The activation of the receptor may be due to an exogenous or endogenous agonist agent, or may be the constitutive activation associated with a native, mutated or modified receptor. The receptor may be purified or present in an in vitro or in vivo system. The receptor may also be present in the central or peripheral nervous system, blood cells or platelets of a nonhuman or human subject. Also provided are kits for performing the same.

[0079] In one embodiment, the compound of formula (I) is selective for 5-HT class serotonin receptors, such as the 5-HT_{2A} subclass of serotonin receptors. In another embodiment, the compound has little or substantially no anti-dopaminergic activity.

[0080] One embodiment provides methods of treating a disease condition associated with a monoamine receptor comprising administering to a mammal in need of such treatment an effective amount of the compound of formula (I). One embodiment provides methods for treating or alleviating disease conditions associated with improper function or stimulation of native, as well as mutated or otherwise modified, forms of central serotonin receptors, particularly the 5-HT class of such receptors, comprising administration of an effective amount of a selective inverse agonist of formula (I) to a host in need of such treatment. Also provided are kits for performing the same.

[0081] In one embodiment, the receptor is the 5-HT_{2A} subclass. In one embodiment, the disease condition is associated with dysfunction of the serotonin receptor. In another embodiment, the disease condition is associated with activation of the serotonin receptor, for instance, inappropriately elevated or constitutive activation, elevated serotonergic tone, as well as disease conditions associated with secondary cellular functions impaired by such pathologies.

[0082] Examples of diseases for which such treatment using the compound of formula (I) is useful include, but are not limited to, neuropsychiatric diseases such as schizophrenia and related idiopathic psychoses, anxiety, sleep disorders, appetite disorders, affective disorders such as major depression, bipolar disorder, and depression with psychotic features, and Tourette's Syndrome, drug-induced psychoses, psychoses secondary to neurodegenerative disorders such as Alzheimer's or Huntington's Disease. It is anticipated that the compound of formula (I), a particularly selective inverse agonist of 5-HT_{2A} that shows little or no activity on dopaminergic receptors, may be especially useful for treating schizophrenia. Treatment using the compound of formula (I) may also be useful in treating migraine, vasospasm, hypertension, various thrombotic conditions including myo-

cardial infarction, thrombotic or ischemic stroke, idiopathic and thrombotic thrombocytopenic purpura, and peripheral vascular disease.

[0083] In a further embodiment the present invention provides methods for treating or alleviating a disease condition associated with improper function, dysfunction, or stimulation of native, as well as mutated or otherwise modified, forms of central or peripheral monoamine receptors, such methods comprising administration of an effective amount of a compound of formula (I) to a host in need of such treatment. In one embodiment, the monoamine receptor is serotonin receptor in the peripheral nervous system, blood or platelets. In some embodiments, the serotonin receptor is a 5-HT_{2A} subclass receptor. In additional embodiments, the disease condition is associated with increased activity or activation of a serotonin receptor. Also provided are kits for performing the same.

[0084] Some embodiments also pertain to the field of predictive medicine in which pharmacogenomics is used for prognostic (predictive) purposes. Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See e.g., Eichelbaum, *Clin Exp Pharmacol. Physiol.*, 23:983-985 (1996), and Linder, *Clin. Chem.* 43:254-66 (1997). In general, two types of pharmacogenetic conditions can be differentiated: genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action), and genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur as naturally occurring polymorphisms.

[0085] One pharmacogenomics approach to identifying genes that predict drug response, known as "a genome-wide association," relies primarily on a high-resolution map of the human genome consisting of already known gene-related markers (e.g., a "bi-allelic" gene marker map that consists of 60,000-100,000 polymorphic or variable sites on the human genome, each of which has two variants). Such a high-resolution genetic map can be compared to a map of the genome of each of a statistically significant number of patients taking part in a Phase II/III drug trial to identify markers associated with a particular observed drug response or side effect. Alternatively, such a high-resolution map can be generated from a combination of some ten-million known single nucleotide polymorphisms (SNPs) in the human genome. As used herein, a "SNP" is a common alteration that occurs in a single nucleotide base in a stretch of DNA. For example, a SNP may occur once per every 1,000 bases of DNA. A SNP may be involved in a disease process; however, the vast majority may not be disease-associated. Given a genetic map based on the occurrence of such SNPs, individuals can be grouped into genetic categories depending on a particular pattern of SNPs in their individual genome. In such a manner, treatment regimens can be tailored to groups of genetically similar individuals, taking into account traits that may be common among such genetically similar individuals.

[0086] Alternatively, a method termed the "candidate gene approach" can be utilized to identify genes that predict drug response. According to this method, if a gene that encodes a drug's target is known (e.g., a protein or a receptor of the

present invention), all common variants of that gene can be fairly easily identified in the population and it can be determined if having one version of the gene versus another is associated with a particular drug response.

[0087] Alternatively, a method termed the “gene expression profiling”, can be utilized to identify genes that predict drug response. For example, the gene expression of an animal dosed with a drug (e.g., a molecule or modulator of the present invention) can give an indication whether gene pathways related to toxicity have been turned on.

[0088] Information generated from more than one of the above pharmacogenomics approaches can be used to determine appropriate dosage and treatment regimens for prophylactic or therapeutic treatment of an individual. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with a molecule or modulator of the invention, such as a modulator identified by one of the exemplary screening assays described herein. As we have described previously, this approach can also be used to identify novel candidate receptor or other genes suitable for further pharmacological characterization *in vitro* and *in vivo*.

[0089] Accordingly, one embodiment provides methods and kits for identifying a genetic polymorphism predisposing a subject to being responsive to the compound of formula (I). The method comprises administering to a subject an effective amount of the compound; identifying a responsive subject having an ameliorated disease condition associated with a monoamine receptor; and identifying a genetic polymorphism in the responsive subject, wherein the genetic polymorphism predisposes a subject to being responsive to the compound. It is anticipated that this method may be useful both for predicting which individuals are responsive to therapeutic effects of the compound and also for predicting those likely to experience adverse side effect responses. This approach may be useful for identifying, for example, polymorphisms in a serotonin receptor that lead to constitutive activation and are thus amenable to inverse agonist therapy. In addition, this method may be useful for identifying polymorphisms that lead to altered drug metabolism whereby toxic byproducts are generated in the body. Such a mechanism has been implicated in the rare, but potentially life threatening side effects of the atypical antipsychotic, clozapine.

[0090] In a related embodiment, a method for identifying a subject suitable for treatment with the compound of formula (I) is provided. According to the method, the presence of a polymorphism that predisposes the subject to being responsive to the compound is detected, the presence of the polymorphism indicating that the subject is suitable for treatment. Also provided are kits for performing the same.

[0091] The compound of formula (I) preferably shows selective inverse agonist activity towards the 5-HT_{2A} receptor. Such activity is defined by an ability of the ligand to attenuate or abolish the constitutive signaling activity of this receptor. Selectivity in the present context is understood as a property of a compound of the invention whereby an amount of compound that effectively inversely agonizes the 5-HT_{2A} receptor and thereby decreases its activity causes little or no inverse agonistic or antagonistic activity at other,

related or unrelated, receptors. In particular, the compound of formula (I) has surprisingly been found not to interact strongly with other serotonin receptors (5-HT 1A, 1B, 1D, 1E, 1F, 2B, 2C, 4A, 6, and 7) at concentrations where the signaling of the 5-HT_{2A} receptor is strongly or completely inhibited. In one embodiment, the compound is also selective with respect to other monoamine-binding receptors, such as the dopaminergic, histaminergic, adrenergic and muscarinic receptors.

[0092] One embodiment of the present invention relates to a method of alleviating or treating a disease condition in which modification of monoamine receptor activity, in particular 5-HT_{2A} serotonergic receptor activity, has a beneficial effect by administering a therapeutically effective amount of the compound of formula (I) to a subject in need of such treatment. Such diseases or conditions may, for instance arise from inappropriate stimulation or activation of serotonergic receptors. It is anticipated that by using a compound that is selective for a particular serotonin receptor subtype, in particular 5-HT_{2A}, the problems with adverse side effects observed with the known antipsychotic drugs, such as extrapyramidal effects, may be avoided substantially.

[0093] The term “therapeutically effective amount” as used herein means an amount of an active compound or pharmaceutical agent that elicits the biological or medicinal response in a tissue, system, animal or human that is being sought by a researcher, veterinarian, medical doctor or other clinician, which includes alleviation, amelioration, or lessening of the symptoms of the disease being treated, or prevents or slows the progress of the disease or increase of the symptoms.

[0094] In one embodiment, the compound of formula (I) may be administered in a single daily dose, or the total daily dosage may be administered in divided doses, for example, two, three or four times daily. Furthermore, the compound of formula (I) may be administered in intranasal form via topical use of suitable intranasal vehicles, via transdermal routes, using those forms of transdermal skin patches well known to persons skilled in the art, by implantable pumps; or by any other suitable means of administration. To be administered in the form of a transdermal delivery system, for example, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

[0095] The dosage regimen utilizing the compound of formula (I) is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the disease or disorder that is being treated.

[0096] For oral administration, compositions containing the compound of formula (I) are preferably provided in the form of tablets containing 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0 or 50.0 mg of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. In one embodiment, a unit dose contains from about

0.001 mg to about 50 mg of the active ingredient. In another embodiment a unit dose contains from about 1 mg to about 10 mg of active ingredient.

[0097] The compound of formula (I) may be used alone at appropriate dosages defined by routine testing in order to obtain optimal pharmacological effect on a monoaminergic receptor, in particular the 5-HT_{2A} serotonergic receptor subtype, while minimizing any potential toxic or otherwise unwanted effects. In addition, co-administration or sequential administration of other agents that improve the effect of the compound may, in some cases, be desirable.

[0098] In one embodiment, the compound of formula (I) may be combined with an additional therapeutic agent. Additional therapeutic agents may include: levodopa (SINEMETTM), SINEMET-CRTM, bromocriptine (PARLODELTM), pergolide (PERMAXTM), ephedrine sulfate (EPHEDRINETM), pemoline (CYLERTTM), mazindol (SANDOZTM), d,1- α -methylphenethylamine (ADDERALLTM), methylphenidate (RITALINTM), pramipexole (MIRAPEXTM), modafinil (PROVIGILTM), ropinirole (REQUIPTM), an anti-dyskinesia agent, an anti-dystonia, an anti-myoclonus, an anti-tremor agent, or an anti-psychotic agent. In some embodiments, the anti-dyskinesia agent is selected from baclofen (LIORESALTM), botulinum toxin (BotoxTM), clonazepam (KlonopinTM), or diazepam (ValiumTM). In some embodiments, the anti-dystonia, anti-myoclonus, or anti-tremor agents are selected from baclofen (LIORESALTM), botulinum toxin (BOTOXTM), clonazepam (KLONOPINTM), or diazepam (VALIUMTM). In some embodiments, the anti-psychotic agent is selected from chlorpromazine (THORAZINETM), haloperidol (HALDOLTM), molindone (MOBANTM), thioridazine (MELLARILTM), a phenothiazine, a butyrophenone, diphenhydramine (pimozide), thioxanthines (flupenthixol), substituted benzamides (sulpiride), sertindole, amisulpride, risperidone, clozapine, olanzapine, ziprasidone, aripiprazole, or their active metabolites (N-desmethylozapine, N-desmethylolanzapine, 9-OH-risperidone)).

[0099] The pharmacological properties and the selectivity of the compound of formula (I) for specific serotonergic receptor subtypes may be demonstrated by a number of different assay methods using recombinant receptor subtypes, preferably of the human receptors if these are available, e.g. conventional second messenger or binding assays. A particularly convenient functional assay system is the receptor selection and amplification assay disclosed in U.S. Pat. No. 5,707,798, which describes a method of screening for bioactive compounds by utilizing the ability of cells transfected with receptor DNA, e.g., coding for the different serotonergic subtypes, to amplify in the presence of a ligand of the receptor. Cell amplification is detected as increased levels of a marker also expressed by the cells.

[0100] Treatment of Neuropsychiatric Disorders

[0101] In one embodiment, the compound of formula (I) and related serotonin 2A and/or 2C receptor inverse agonists alone or in combination with other antipsychotic drugs, particularly those with dopamine antagonist properties, are used to treat a variety of human neuropsychiatric diseases including schizophrenia, schizoaffective disorders, mania and psychotic depression. Specifically, the compound of formula (I) and related serotonin 2A/2C receptor inverse agonists can improve psychotic symptoms (feelings of being

controlled by outside forces, hearing, seeing, smelling or feeling things which are not there, hallucinations and unusual beliefs, delusions), negative symptoms (loss of normal behavior including tiredness, loss of concentration and lack of energy and motivation, and cognitive function in psychotic patients when used alone or in combination with other antipsychotic drugs. These agents also reduce the side-effects associated with the use of existing antipsychotic drugs and reduce the dose of existing agent that is required to achieve antipsychotic efficacy. Specifically, the compound of formula (I) and related compounds alone or in combination with existing antipsychotic drugs can be used to control the behavioral and neuropsychiatric manifestations present in all of these disease states. In some embodiments, pharmaceutical compositions comprised of a combination of the compound of formula (I) and existing antipsychotic agents are used.

[0102] Neuropsychiatric disorders associated with psychosis affect a large proportion of the human population. Psychosis appears as a dominating symptom in diverse disorders, including schizophrenia, schizoaffective states, mania, psychotic depression among others. Current treatment options primarily involve pharmacotherapy with a class of drugs known as antipsychotics. Antipsychotics are effective in ameliorating positive symptomatology of these disorders, yet they frequently do not improve and may worsen negative and cognitive symptoms. Significant treatment limiting side effects are common with the use of antipsychotic drugs.

[0103] Drugs that possess antipsychotic properties have been in clinical use since the early 1950's. Antipsychotic drugs are widely prescribed to treat psychotic symptoms irrespective of their etiology. Clinical use of these compounds is limited, however, by their side effect profiles. Nearly all of the "typical" or first generation compounds have significant adverse effects on human motor function. These "extrapyramidal" side effects, so termed due to their effects on human motor systems, can be both acute and chronic in nature. Acute effects include dystonic reactions, and a potentially life threatening but rare symptom constellation; neuroleptic malignant syndrome. Chronic side effects include akathisia, tremors, and tardive dyskinesia. Due in large part to these disabling side effects, antipsychotic drug development has been focused on newer "atypical" agents (clozapine, olanzapine, quetiapine, risperidone, aripiprazole) that appear to have reduced liability for inducing adverse motoric effects. These newer "atypical" antipsychotic drugs, however, suffer from other limiting side-effects, including induction of cardiovascular abnormalities, extreme sedation, morbid obesity, type II diabetes, blood dyscrasias and pancreatitis among others.

[0104] While the precise molecular mechanisms mediating antipsychotic drug action remain to be elucidated, antipsychotic drugs have been shown, by both in vitro and in vivo methods, to interact with a large number of central monoaminergic neurotransmitter receptors, including dopaminergic, serotonergic, adrenergic, muscarinic, and histaminergic receptors. It is likely that the therapeutic and adverse effects of these drugs are separable and are mediated by distinct receptor subtypes.

[0105] Currently, it is thought that antipsychotic drugs reduce the positive symptoms in these disorders by blocking

dopamine D2 receptors. This is based on the observation that these all antipsychotic drugs have reasonable affinity for this receptor in vitro, and that a correlation exists between their potency to block D2 receptors and their ability to reduce the positive symptoms of these disorders. Unfortunately, it is likely that antagonism of dopamine D2 receptors also mediates the disabling extrapyramidal side effects.

[0106] The only other consistent receptor interaction that these drugs as a class display is inverse agonism of 5-HT_{2A} receptors, suggesting that inverse agonism of these receptors is an alternate molecular mechanism that confers antipsychotic efficacy. This theory is bolstered by a number of basic scientific and clinical observations regarding serotonergic systems and the 5-HT_{2A} receptor in particular (U.S. Pat. No. 6,358,698 incorporated by reference).

[0107] However, nearly all known antipsychotic agents lack specificity in their mechanisms of action. In addition to possessing activity at dopamine D2 receptors and 5-HT_{2A} receptors, these drugs as a class have a multitude of pharmacologically relevant interactions with critical neuronal proteins including a host of cell surface receptors, ion channels, and re-uptake transporters. This lack of drug target specificity likely contributes to the multiplicity of adverse effects associated with use of existing antipsychotic agents.

[0108] These observations highlight the need to develop novel therapeutic regimens that are specifically designed to not only demonstrate efficacy against these particular disabling symptoms but to also possess tolerability in these specific patient populations. This can be achieved by improving the selectivity of the drug target interactions of new therapeutic agents. Specifically, the development of agents with novel mechanisms of action that avoid the known pitfalls associated with existing agents is desired. In addition, improved selectivity avoids the known adverse effects associated with interactions with non-efficacy off-target receptor interaction. For example many antipsychotic drugs possess high affinity interactions with H₁ receptors. H₁ antagonism is associated with sedation. Further, other antipsychotic drugs have affinity interactions with alpha receptors. Antagonism of alpha-1 receptors is associated with orthostasis. Improvements in therapeutic efficacy and safety also can be achieved by combining two or more agents each with selective target interactions to achieve additive or synergistic benefits. Specifically, by combining one drug that specifically interacts with D2 receptors as an antagonist and another drug like the compound of formula (I) that interacts with specifically with 5-HT_{2A/2C} receptors as antagonist or inverse agonist, the multitude of off-target interactions of existing antipsychotic drugs can be avoided.

[0109] In one embodiment, serotonin 2A and/or 2C receptor inverse agonists are used to treat a variety of human neuropsychiatric diseases including schizophrenia, schizoaffective disorders, mania, behavioral disturbances associated with dementia and psychotic depression. For example, the compounds disclosed herein have utility in reducing the positive symptoms, improving negative symptoms and enhancing cognitive function in patients with certain neuropsychiatric diseases.

[0110] Antipsychotics and dopamine receptor antagonists can be effective in ameliorating positive symptoms in schizophrenia and related diseases. Unfortunately, many of these compounds significantly worsen motor function and

increase negative symptoms or leave these and other symptoms untreated in these patients. Biochemical and pharmacological data support the hypothesis that potentiation of serotonergic neurotransmission may be pathophysiologically important in the development of these unwanted effects and conversely blockade of serotonergic neurotransmission may reduced the side-effects associated with antipsychotic drug therapy. While not being bound by this theory, the compound of formula (I) was selected to exploit the relationship of serotonergic activity and the limiting effects associated with antipsychotic therapy.

[0111] Haloperidol is a typical antipsychotic with specificity as a D2 receptor antagonist. This compound commonly is used to treat the positive symptoms associated with acute exacerbations of schizophrenia. Unfortunately, the use of this compound is associated with a plethora of unwanted motoric side effects, including akathisia, parkinsonism, tardive dyskinesia and neuroleptic malignant syndrome. This compound also does not alter or worsens negative symptoms and cognitive function in these patients.

[0112] In one embodiment, the compound of formula (I) can be used to treat many side-effects that arise from antipsychotic therapy. For example, the compound of formula (I) may be useful for treatment of motoric side-effects of other antipsychotic agents such as haloperidol. In one embodiment, the compound of formula (I) is used for the treatment of motoric side-effects associated with haloperidol treatment.

[0113] In one embodiment, the compound of formula (I) may be used prophylactically when for example, it is considered necessary to initiate haloperidol therapy and it is feared that motoric deficits may develop.

[0114] In some embodiments, the compound of formula (I) may be used to treat psychosis as a monotherapy or as an adjunct to medicaments to prevent or treat antipsychotic drug side-effects caused by the medicament. Alternatively, the compound of formula (I) may be given in combination with other compounds, which also reduce antipsychotic drug side-effects.

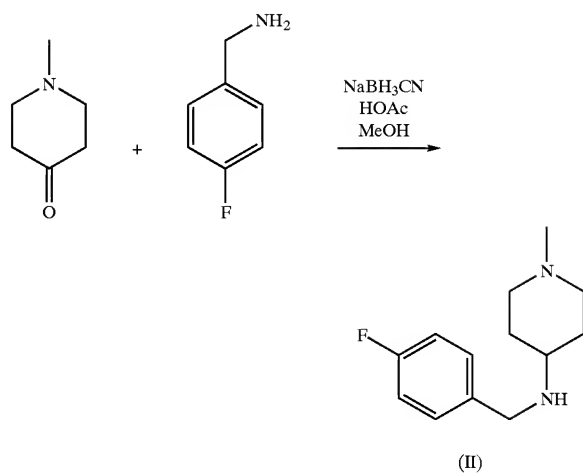
[0115] In one embodiment, the compound of formula (I) may be used to treat the negative symptoms of certain neuropsychiatric disease including schizophrenia as a monotherapy or as an adjunct to medicaments used to treat the positive symptom of these diseases.

[0116] In some embodiments, the compound of formula (I) also may be used to improve cognitive function in certain neuropsychiatric disease including schizophrenia as a monotherapy or as an adjunct to medicaments used to treat the positive symptom of these diseases.

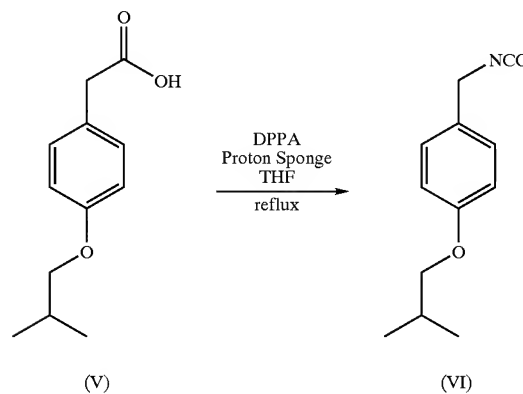
[0117] Methods of Preparation

[0118] The compound of formula (I) may be synthesized by methods described below, or by modification of these methods. Ways of modifying the methodology include, among others, modification in temperature, solvent, reagents, etc.

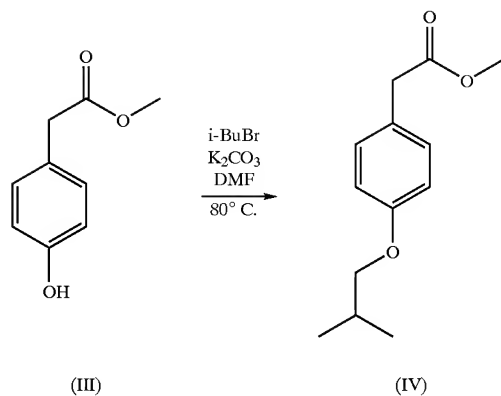
[0119] The first step of the synthesis, illustrated below, is conducted in the presence of acetic acid, NaBH₃CN, and methanol to produce the compound of formula (II):



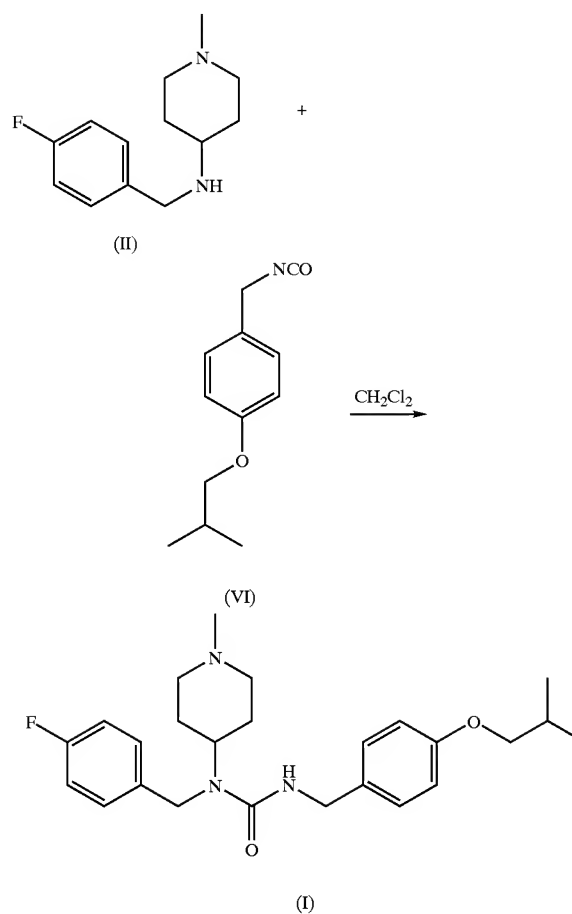
[0122] The compound of formula (V) is heated to reflux with diphenylphosphonyl azide (DPPA) and a proton sponge in tetrahydrofuran (THF) to produce the compound of formula (VI):



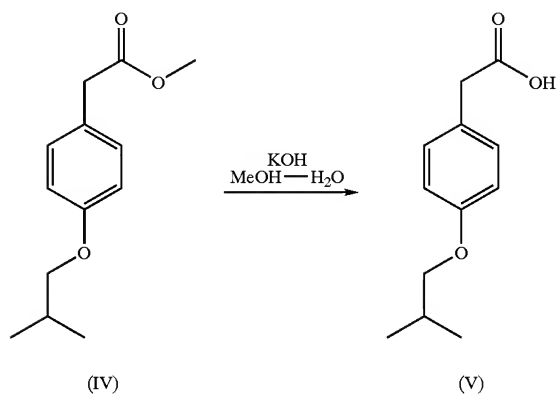
[0120] The compound of formula (IV) can be synthesized by treatment of the compound of formula (III) with isobutyl bromide and potassium carbonate in dimethyl formamide (DMF) at about 80°C :



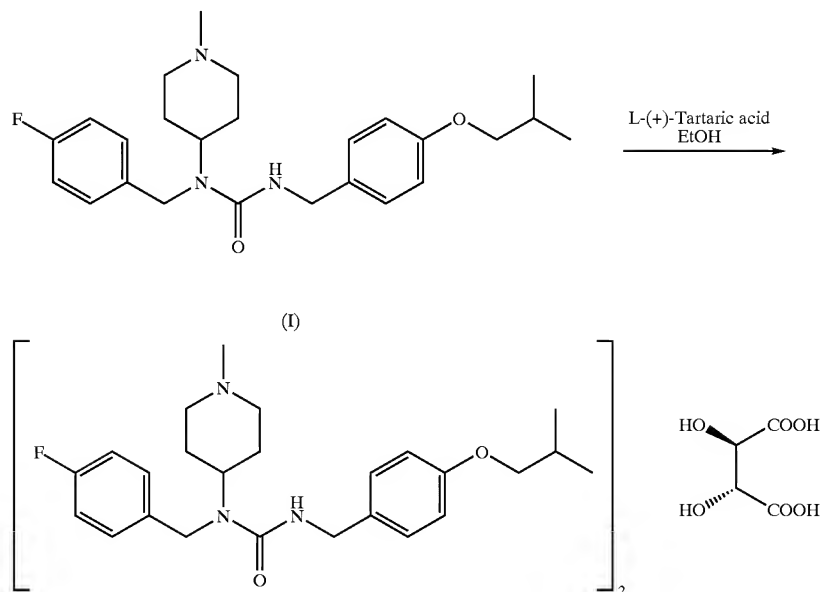
[0123] Finally, reaction of the compound of formula (II) with the compound of formula (VI) in methylene chloride produces the compound of formula (I):



[0121] The compound of formula (IV) can be converted to the compound of formula (V) by reaction with potassium hydroxide in methanol/water:



[0124] The tartrate salt of the compound of formula (I) may be produced by mixing with L-(+)-Tartaric acid in ethanol:



EXAMPLES

[0125] The examples below are non-limiting and are set forth to illustrate some of the embodiments disclosed herein.

Example 1

Agonist Studies

[0126] Parkinson's disease is typically managed using direct acting dopamine agonists. Examples of this class of compounds include pergolide, bromocriptine, pramipexole and ropinirole. These drugs are thought to be effective because of their agonist activity at the dopamine D₂, D₃, and D₄ receptors located in striatal and forebrain regions. This activity may compensate for the progressive loss of fore-brain dopaminergic innervation that characterizes the PD. However, these drugs are not specific for these dopaminergic receptors and also possess potent agonist activity at other receptors, including 5HT_{2A} and 5HT_{2C} receptors. Using a physiologically predictive in vitro functional assay, it is shown below that pergolide, lisuride, and bromocriptine display agonist potencies at human 5HT_{2A} receptors that are equivalent to those observed at the human D₂ receptor. (FIGS. 1A and 1B, and Table 1).

[0127] Using the R-SAT assay, the activity of common dopaminergic compounds against dopamine and serotonin receptor types was studied. (See U.S. Pat. Nos. 5,912,132 and 5,955,281, both of which are hereby incorporated by reference.) In FIG. 1, data were plotted as percentage agonist response as determined for a reference full agonist (100%) versus drug concentration. The reference full agonist used for the D₂ receptor was quinpirole, while serotonin was used for the 5HT_{2A} receptor. Compounds tested include dopamine (filled squares), quinpirole (filled circles), lisuride

(filled triangles), bromocriptine (filled diamonds), serotonin (open squares), and pergolide (filled inverted triangles). Potencies of representative dose response curves using dopamine D₂ receptors were determined and are shown in FIG. 1A; (pergolide-0.21 nM, dopamine-8.0 nM, lisuride-0.023 nM, quinpirole-3.3 nM, bromocriptine-0.43 nM, and serotonin-no response). FIG. 1B shows compound potency against the serotonin 5-HT_{2A} receptor; (dopamine-no response, quinpirole-174 nM, lisuride-0.028 nM, bromocriptine-2.7 nM, serotonin-33 nM, and pergolide-0.22 nM).

[0128] Because these drugs are administered in the clinic to achieve D₂ receptor occupancy, these data argue that direct acting dopamine agonists are also behaving as 5HT_{2A} receptor agonists in vivo when administered in therapeutic doses to PD patients.

TABLE 1

Serotonin Receptor Agonist Activity of Dopaminergic Agents Used in PD			
Drug	Dopamine D2	Serotonin 2A	Serotonin 2C
Dopamine	8.40 +/- 0.32	NA	NA
Serotonin	NA	7.73 +/- 0.04	7.29 +/- 0.10
Lisuride	11.00 +/- 0.36	10.65 +/- 0.10	7.61 +/- 0.13
Pergolide	9.45 +/- 0.06	8.05 +/- 0.22	6.66 +/- 0.08
Bromocriptine	9.30 +/- 0.31	8.75 +/- 0.14	5.80 +/- 0.05
Ropinirole	8.19 +/- 0.58	6.85 +/- 0.77	NT
Pramipexole	8.15 +/- 0.38	5.93 +/- 0.74	NT
Apomorphine	6.24 +/- 0.11	NA	NA

[0129] Data are derived from R-SAT assays. As shown, all compounds displayed full (>75%) relative agonist efficacies. Data are reported as -Log (EC₅₀) values +/- standard deviation of three to eight separate determinations. The

VGV isoform of the 5HT_{2C} receptor, and the short form of the D₂ receptor were utilized for these studies. NA denotes no activity, NT denotes not tested.

[0130] The agonist activity of these anti-parkinsonian agents at human 5HT_{2A/C} receptors has particular implications for the generation and treatment of human hallucinations and psychosis. That certain natural and synthetic chemical compounds can induce hallucinatory states in humans has led to detailed investigations of the mechanisms of action of these hallucinogenic or psychotomimetic drugs. These efforts have implicated a number of molecular activities of these classes of drugs as being relevant to their ability to induce hallucinations, particularly visual hallucinations, in normal healthy individuals. Hallucinogens fall into two distinct chemical classes, the phenylethanolamines, and the substituted tryptamines, both of which are structurally related to serotonin. Many in vitro studies, utilizing radioligand binding techniques, as well as functional pharmacological assays, have repeatedly demonstrated that these drugs are potent 5HT_{2A} and 5HT_{2C} receptor agonists (5). More recent in vivo studies, in which normal volunteers are administered the hallucinogen MDMA (Ecstasy) and then evaluated for clinical response, as well as anatomical measures of brain activation utilizing functional neuro-imaging technologies, have demonstrated that the psychometric and pharmacological activities of hallucinogens can be blocked by anti-psychotic drugs as well as the compound ketanserin (6,7). These drugs share a common molecular property, 5HT_{2A} receptor inverse agonism.

Example 2

Inverse Agonist Studies

[0131] Once treatment-induced motoric and neuropsychiatric symptoms develop in PD patients, few viable therapeutic options exist to manage these disturbances. Treatment strategies differ for these two classes of symptoms, but one uniformly clinically efficacious, yet poorly tolerated approach, involves the use of antipsychotic agents. Antipsychotics are known to possess high affinity for the dopamine D₂ subclass of dopamine receptors and neutral antagonism of these receptors underlie the therapeutic efficacy of these drugs in human psychosis. In addition to dopamine D₂ receptor antagonism, these agents possess a wide range of additional potent and pharmacologically relevant activities at many of the other monoaminergic receptor subtypes including serotonin, adrenergic, muscarinic and histaminergic receptors. Of these additional molecular actions, 5HT_{2A} receptor interactions have been the subject of significant study. That antipsychotics have high affinity for multiple receptor subtypes, including serotonin 2 receptors, was demonstrated by the application of radioligand binding techniques (8). The methodologies used to document this cannot define the nature of the interaction between an anti-psychotic antipsychotic and a given receptor. For example, the methods are unable to distinguish as to whether a drug possesses positive (agonist) or negative (inverse agonist) intrinsic activity, or if it lacks intrinsic activity and functions as a neutral antagonist. Recently, this class of drugs was profiled using a functional assay that can discriminate the mechanistic nature of a drug-target interaction (9).

[0132] This approach revealed a number of novel aspects of antipsychotic drug action (See U.S. Pat. No. 6,358,698,

which is hereby incorporated by reference in its entirety). It confirmed that these drugs as a class possess potent neutral antagonistic activity at the D₂ receptor. Importantly, it also revealed that nearly all antipsychotic drugs, with the exception of the substituted benzamides, possess potent negative intrinsic activity (inverse agonism) at the 5HT_{2A} receptor. These efforts have identified inverse agonist activity at the 5HT_{2A} receptor as being a critical molecular component of anti-psychotic drug action, and suggest that compounds that are selective 5HT_{2A} receptor inverse agonists may have antipsychotic efficacy, even in the absence of D₂ receptor activity.

[0133] None of the older typical antipsychotics, exemplified by haloperidol, can be administered to PD patients because of severe worsening in their motor states. The more recent development of newer atypical agents, namely those with reduced (but clearly not absent) liability to induced motoric side effects, suggested that perhaps these agents could be used in PD patients to control dyskinesias and hallucinosis. Unfortunately, the majority of these agents are not tolerated in PD patients secondary to worsening of motor function (10). Of the atypical agents, only one, clozapine, has shown efficacy in treating these adverse treatment-induced side effects in PD patients without untoward motoric liabilities. As such, an improved understanding of the in vitro molecular profile of clozapine can provide critical insights into the design of novel agents for these difficult to treat indications.

[0134] The demonstration that clozapine is tolerated in PD patients comes from studies on treatment-induced psychosis. Two well-designed placebo controlled, double blind clinical trials have shown that clozapine is efficacious in psychotic PD patients, and does not worsen parkinsonism, at doses in the 25-35 mg/day range (11,12). Similarly, two open label studies of clozapine in L-dopa and apomorphine induced dyskinesias also demonstrate efficacy and tolerability of low doses of clozapine, on the order of 50-100 mgs/day in these patients (13,14). The dosages used in these PD patients are much lower than the typical 600-900 mg/day range of doses used in treatment refractory schizophrenia. Commensurate with this lower dosing, plasma levels of clozapine in PD patients with psychosis ranged from 4.5 to 16.1 ng/ml (15). This is dramatically lower than the ≥ 250 ng/ml average serum levels that are associated with therapeutic response in refractory schizophrenic patients.

[0135] Not surprisingly, the administration of low dose clozapine, and the commensurate plasma levels obtained at these doses, are well below those necessary for D₂ receptor occupancy, providing a mechanistic understanding of why these dosages are tolerated with respect to motoric liability in these patients. (Positron emission tomography (PET) studies in schizophrenic patients have defined steady state plasma concentrations of clozapine that are required to generate high occupancy of striatal dopamine D₂ receptors). These data also argue that efficacy in dyskinesia and psychosis is mediated by one or more of the non-D₂ receptor targets of this drug. Since rank orders of receptor potencies, as determined by in vitro pharmacological assays, has repeatedly been shown to be a reliable predictor of in vivo receptor action, the receptor sites for which clozapine display a higher potency than D₂ receptors would be predicted to potentially mediate its clinical efficacy in this indication. Detailed functional profiling of clozapine against over 30 of

the known monoaminergic receptor subtypes has identified only five sites with higher affinity than dopamine D₂ receptors, histamine H₁, muscarinic m1 and m4, and serotonin 2A, 2B, and 6 receptors. Table 2 reports the absolute and relative potencies of clozapine at some of these monoamine receptor targets as determined by the physiologically predictive in vitro R-SAT assay. These data suggest that at the clinical dosing and serum levels of clozapine observed in PD, two receptor sites are preferentially occupied, the histamine H₁ and 5HT_{2A} receptors.

[0136] Conversely, plasma levels achieved with 50 mgs/day of clozapine result in full occupancy of cortical 5HT_{2A} receptors, and extrapolation to the plasma levels observed in PD patients treated for psychosis suggest near complete occupancy of 5HT_{2A} receptors at these dosages as well (16). Whereas central occupancy of 5HT_{2A} receptors, coupled with negative intrinsic activity, may mediate efficacy in these states, central occupancy of histamine H₁ receptors is known to cause sedation, an effect that was observed in the majority of PD patients treated with low dose clozapine. Taken together these data suggest that clozapine is acting primarily as a 5HT_{2A} receptor inverse agonist in this clinical setting.

TABLE 2

Antagonist and Inverse Agonist Potencies of Clozapine at Monoamine Receptors					
	D ₂	5HT _{2A}	5HT _{2B}	5HT _{2C}	H ₁
Clozapine	72 +/- 56	6.4 +/- 1.0	20 +/- 9	250 +/- 60	0.40 +/- 0.07
Ratio to D ₂		11	3.6	0.3	180

[0137] Data are derived from (9) and are reported as Ki values for the D₂ receptor determined as a competitive antagonist, and EC₅₀ values for the remaining receptors determined as inverse agonists, in nanomolar unit's +/- standard deviation of three to eight separate determinations.

[0138] Behavioral Pharmacological Evidence

[0139] The tartrate salt of the compound, N-(1-methylpiperidin-4-yl)-N-(4-fluorophenylmethyl)-N'-(4-(2-methylpropyloxy)phenylmethyl)carbamide (compound of formula (I)), is a potent, selective, orally bioavailable 5HT_{2A} receptor inverse agonist. The compound of formula (I) also possesses lesser potency as a 5-HT_{2C} receptor inverse agonist and lacks intrinsic activity at the remaining monoaminergic receptor subtypes. Perhaps most notably, the compound of formula (I) lacks activity at dopamine receptor subtypes. (See U.S. patent application Ser. No. 09/800,096, which is hereby incorporated by reference in its entirety). Extensive behavioral pharmacological profiling of this agent, including pre-clinical models of antipsychotic and anti-dyskinetic drug actions support the therapeutic use of the compound of formula (I) in Parkinson's Disease and related human neurodegenerative diseases.

Example 3

Animal Studies

[0140] To determine potential in vivo antipsychotic activity, we studied the compound of formula (I) in an animal

model that predicts such efficacy in humans. The compound of formula (I) attenuates hyperactivity induced by the non-competitive N-methyl-d-aspartate (NMDA) antagonist MK-801 (dizocilpine) with a minimum effective dose of 1 mg/kg s.c. (FIG. 2A), and 10 mg/kg p.o. (FIG. 2B). The compound of formula (I) also reduced spontaneous locomotion at 3 mg/kg and higher s.c. doses (FIG. 2A), and at oral doses between 10 and 100 mg/kg (FIG. 2B). In FIG. 2A and 2B, asterisks indicate statistical significance (p<0.05) compared to respective vehicle control. Inhibition of MK-801 is a property shared by most atypical antipsychotic agents, and after i.p. administration, the compound of formula (I) attenuated MK-801 hyperactivity at 1 mg/kg, in a manner similar to the atypical antipsychotic clozapine.

Example 4

Primate Animal Studies

[0141] To determine the potential in vivo anti-dyskinetic activity, we studied the compound of formula (I) in an animal model that predicts such efficacy in humans. The use of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to induce parkinsonism in monkeys, coupled with prolonged administration of L-dopa induces severe dyskinesias. The compound of formula (I), when administered s.c., to dyskinetic primates was found to significantly diminish L-dopa induced dyskinesias in a dose dependent manner as determined by the reduction of observable dyskinetic movements scored as a percentage of those present in placebo injected animals (FIG. 3).

Example 5

5HT_{2A/C} Serotonin Antagonist Treatment of Parkinson's Disease

[0142] The present example demonstrates that blockage of 5HT_{2A/C} receptors with the compound of formula (I) in parkinsonian patients reduces levodopa-associated dyskinesias and motor response fluctuations. Additionally, the compound of formula (I) is shown to be safe and tolerated at effective doses and potentiates the beneficial effects of levodopa on parkinsonian symptoms.

[0143] The compound of formula (I) is administered orally in a group of 21 parkinsonian patients in a double blind, placebo controlled study lasting approximately 5 weeks. An unbalanced parallel-group dose escalation design is used involving an initial placebo run-in, followed by a randomized (active) phase of the compound of formula (I) or placebo. The compound of formula (I) is administered once daily for four weeks, with the dose escalating once each week. Assessments are made on the first day of each dose escalation.

[0144] The study is conducted on an outpatient basis. Studies of the compound of formula (I) effect on the motor response to levodopa are conducted in accordance with the standard Experimental Therapeutics Branch (ETB) paradigm, which makes use of a steady state infusion of dopaminomimetics in order to maximize the reliability of data acquisition as well as to permit determination of the anti-parkinsonian efficacy half-time.

[0145] Patients who participate in the study have particular characteristics. The patients are between 30 and 80 years of age, inclusively. The patients had been diagnosed with idiopathic Parkinson's disease based on the presence of a characteristic clinical history and neurological findings. The patients displayed relatively advanced disease symptoms with levodopa-associated motor response complications, including peak-dose dyskinesias and wearing-off fluctuations.

[0146] The sample size is calculated for the primary endpoint: the Unified Parkinson's Disease Rating Scale (UPDRS) part III motor examination. A sample size of 17 provides 80% power to detect predicted differences, a 40% reduction, with a standardized effect size of 1, using a two-tailed t-test at the 0.05 significance. This assumes an anti-dyskinetic effect of the compound of formula (I) to be compared to that of amantadine (as observed in previous ETB studies), and a linear dose-response of the compound of formula (I). In this phase 2 study we will accept a two-sided alpha at a 0.05 significance level. Four patients will be added for the placebo group, totaling 21 subjects enrolled in the study.

[0147] Patients enter the levodopa infusion optimal rate determination (dose finding) portion of the study as soon as all prohibited medication has been withdrawn for at least four weeks. If the patient has had an intravenous dosing rate for levodopa optimized within the past three months, these doses may be used for the study.

[0148] Intravenous infusion of levodopa is conducted in an in-patient ward. On the night prior to all infusions, subjects' usual anti-parkinsonian medications are withheld (levodopa by 12 AM, dopamine agonists by 6 PM). During the first and second days of optimal rate determination, two baseline UPDRS ratings are performed prior to levodopa infusion. Initially, the "optimal" rate of levodopa infusion is carefully titrated for each individual to determine the minimum dose needed to achieve a stable "on" state characterized by an "optimal" reduction in parkinsonian signs and mild but ratable dyskinesias (comparable to patient's usual "on" state). Dyskinesia severity is similar to that experienced with each patient's usual therapeutic regimen. Levodopa will be administered by means of an indwelling intravenous catheter. The initial infusion rate of levodopa will not exceed 80 mg/hr. Subsequent infusion rates may be gradually increased until the optimal rate is found, up to a maximum of 2 mg/kg/hour.

[0149] Levodopa infusions will ordinarily last up to 8 hours, but may be continued uninterrupted for several days or be repeated on other days to obtain reliable assessment of motor function. The peripheral decarboxylase inhibitor carbidopa (50 mg, given every 3 hours) is administered orally starting at least one hour prior to intravenous administration of levodopa and continuing until levodopa effects have worn off. After the initial "optimal" rate finding for levodopa infusion, all subsequent infusions are given at the predetermined "optimal rate". As an intravenous levodopa formulation is not commercially available in this country, is administered under ETB IND 22,663.

[0150] Patients are dosed according to Table 3:

TABLE 3

Patient group	Week 1	Week 2	Week 3	Week 4	Week 5
I	Placebo	Placebo	Placebo	Placebo	Placebo
II	Placebo	30 mg Compound (I)	70 mg Compound (I)	150 mg Compound (I)	300 mg Com- pound (I)

[0151] Patients proceed through this dose escalation scheme until week 5 or until maximum tolerated dose is attained.

[0152] Throughout the study, patients are evaluated weekly for drug safety and tolerability during their inpatient admission and two weeks after treatment for an outpatient follow-up visit. During each inpatient admission, patients remain under close medical monitoring by staff physicians and nurses. If, at any time during the treatment period, the staff physician determines that a patient does not tolerate any given dose, the patient will be considered to have attained maximum tolerated dose and will not receive any additional doses of the compound of formula (I). Patients are encouraged to contact study staff between study days to report any adverse experiences.

[0153] Patients are observed in the hospital and will not be discharged until free of all significant adverse effects, if any. Safety assessments, which are performed on study days, include adverse experiences, monitoring vital signs, standard safety monitoring, and cardiac monitoring.

[0154] Subjects in Patient Group II show a reducing in levodopa-associated dyskinesias and motor response fluctuations. The subjects in Patient Group II tolerate the compound of formula (I) at all doses administered. The compound of formula (I) therapy also potentiates the beneficial effects of levodopa on parkinsonian symptoms.

Example 6

R-SAT Assay

[0155] The functional receptor assay Receptor Selection and Amplification Technology (R-SAT) was used to investigate the activity of the compound of formula (I) as an inverse agonist at 5HT_{2A} receptors. The compound of formula (I) exhibited high potency (pIC₅₀ of 9.1) and high efficacy (98%) at 5HT_{2A} receptors.

Example 7

Anti-Psychotic Activity Study

[0156] To determine potential in vivo antipsychotic activity, we studied the compound of formula (I) in an animal model that predicts such efficacy against positive symptoms in humans (FIG. 4). In FIG. 4, ACP refers to the compound of formula (I). The compound of formula (I) did not reduce hyperactivity induced by 3.0 mg/kg I.P. of the indirect dopamine agonist d-amphetamine when administered alone at doses of 10.0 mg/kg P.O. and below to mice. As expected, haloperidol dose-dependently reduced amphetamine hyperactivity with a minimally significant effect seen at 0.1 mg/kg, s.c. When a 10.0 mg/kg P.O. dose of the compound

of formula (I) was administered in combination with various s.c. doses of haloperidol, the minimally significant dose of haloperidol was decreased to 0.03 mg/kg. With this combination, amphetamine hyperactivity is completely reversed. Thus, an inactive dose of the compound of formula (I), when combined with an inactive dose of haloperidol produces a complete reversal of amphetamine hyperactivity. This suggests that the antipsychotic activity of haloperidol may be significantly enhanced when it is combined with the compound of formula (I). Equally important, when the compound of formula (I) is combined with haloperidol, the dose of haloperidol can be lowered without a loss of efficacy. This would be expected to improve the safety margin for the clinical use of haloperidol in neuropsychiatric diseases.

[0157] Literature Cited

[0158] The following references are incorporated herein by reference in their entireties.

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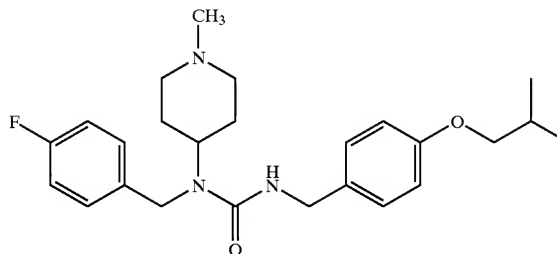
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What is claimed is:

1. A composition comprising a compound of Formula (I):

(I)



and a pharmaceutically acceptable carrier.

2. The composition of claim 1, further comprising an additional therapeutic agent.

3. The composition of claim 2, wherein the additional therapeutic agent is selected from the group consisting of levodopa (SINEMET™, SINEMET-CR™, bromocriptine (PARLODEL™), pergolide (PERMAX™), ephedrine sulfate (EPHEDRINE™), pemoline (CYLERT™), mazindol (SANOREX™), d,l-α-methylphenethylamine (ADDER-

ALL™), methylphenydate (RITALIN™), pramipexole (MIRAPEX™), modafinil (PROVIGIL™), and ropinirole (REQUIP™).

4. The composition of claim 2, wherein the additional therapeutic agent is an anti-dyskinesia agent

5. The composition of claim 2, wherein the additional therapeutic agent is an anti-dyskinesia agent selected from the group consisting of baclofen (LIORESAL™), botulinum toxin (BOTOX™), clonazepam (KLONOPIN™), and diazepam (VALIUM™).

6. The composition of claim 2, wherein the additional therapeutic agent is an anti-dystonia, anti-myoclonus, or anti-tremor agent selected from the group consisting of baclofen (LIORESAL™), botulinum toxin (BOTOX™), clonazepam (KLONOPIN™), and diazepam (VALIUM™).

7. The composition of claim 2, wherein the additional therapeutic agent is an anti-psychotic agent with dopaminergic receptor antagonism.

8. The composition of claim 2, wherein the additional therapeutic agent is an anti-psychotic agent selected from the group consisting of chlorpromazine (THORAZINE™), haloperidol (HALDOL™), molindone (MOBAN™), thioridazine (MELLARIL™), a phenothiazine, a butyrophenone, diphenylbutylpiperidine (pimozide), thioxanthines (flupenthixol), substituted benzamides (sulpiride), sertindole, amisulpride, risperidone, clozapine, olanzapine, ziprasidone, aripiprazole, and their active metabolites (N-desmethylozapine, N-desmethyloanzapine, 9-OH-risperdone)).

9. A method for treating a neurodegenerative disease comprising:

identifying a patient suffering from a neurodegenerative disease; and

administering to the patient an effective amount of an inverse agonist selective for a serotonin receptor; whereby the dopaminergic therapy associated dyskinesia is reduced.

10. The method of claim 9 wherein the neurodegenerative disease is selected from the group consisting Parkinson's disease, Huntington's disease, Alzheimer's disease, Spinocerebellar Atrophy, Tourette's Syndrome, Friedrich's Ataxia, Machado-Joseph's disease, Lewy Body Dementia, Dystonia, Progressive Supranuclear Palsy, and Frontotemporal Dementia

11. The method of claim 9, wherein the serotonin receptor is a 5HT_{2A} receptor.

12. The method of claim 9, wherein the serotonin receptor is a 5HT_{2C} receptor.

13. The method of claim 9, wherein the inverse agonist binds to a 5HT_{2A} receptor or a 5HT_{2C} receptor.

14. The method of claim 9, wherein the inverse agonist is the compound of formula (I).

15. The method of claim 9, further comprises administering a dopaminergic agent in combination with the compound of formula (I).

16. The method of claim 9, wherein the reagent increases dopaminergic activity and is selected from the group consisting of levodopa, SINAMET™, SINAMETCR™, bromocriptine (PARLODEL™), pergolide (PERMAX™), ephedrine sulfate (EPHEDRINE™), pemoline (CYLERT™), mazindol (SANOREX™), d,1- α -methylphenethylamine (ADDERALL™), methylphenydate

(RITALIN™), pramipexole (MIRAPEX™), modafinil (PROVIGIL™), and ropinirole (REQUIP™).

17. A method for treating dyskinesia associated with dopaminergic therapy comprising:

identifying a patient suffering from dopaminergic therapy associated dyskinesia;

administering to the patient an effective amount of an inverse agonist selective for a serotonin receptor; whereby the dopaminergic therapy associated dyskinesia is reduced.

18. The method of claim 17, wherein the serotonin receptor is a 5HT_{2A} receptor.

19. The method of claim 17, wherein the serotonin receptor is a 5HT_{2C} receptor.

20. The method of claim 17, wherein the inverse agonist binds to a 5HT_{2A} receptor and a 5HT_{2C} receptor.

21. The method of claim 17, wherein the inverse agonist is the compound of formula (I).

22. The method of claim 21, further comprising administering an anti-dyskinesia agent in combination with the compound of formula (I).

23. The method of claim 22, wherein the anti-dyskinesia agent is selected from the group consisting of baclofen (LIORESAL™), botulinum toxin (BOTOX™), clonazepam (KLONOPIN™), and diazepam (VALIUM™).

24. The method of claim 17, wherein the patient suffers from a neurodegenerative disease selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, Spinocerebellar Atrophy, Tourette's Syndrome, Friedrich's Ataxia, Machado-Joseph's disease, Lewy Body Dementia, Dystonia, Progressive Supranuclear Palsy, and Frontotemporal Dementia.

25. A method for treating dystonia, myoclonus, or tremor associated with dopaminergic therapy comprising:

identifying a patient suffering from dopaminergic therapy associated dystonia, myoclonus, or tremor;

administering to the patient an effective amount of an inverse agonist selective for a serotonin receptor; whereby the dopaminergic therapy associated dystonia, myoclonus, or tremor is reduced.

26. The method of claim 25, wherein the serotonin receptor is a 5HT_{2A} receptor.

27. The method of claim 25, wherein the serotonin receptor is a 5HT_{2C} receptor.

28. The method of claim 25, wherein the inverse agonist binds to a 5HT_{2A} receptor and a 5HT_{2C} receptor.

29. The method of claim 25, wherein the inverse agonist is the compound of formula (I).

30. The method of claim 29, further comprising an anti-dystonia, anti-myoclonus, or anti-tremor agent in combination with the compound of formula (I).

31. The method of claim 30, wherein the anti-dystonia, anti-myoclonus, or anti-tremor agent is selected from the group consisting of baclofen (LIORESAL™), botulinum toxin (BOTOX™), clonazepam (KLONOPIN™), and diazepam (VALIUM™).

32. A method for treating psychosis associated with dopaminergic therapy comprising:

identifying a patient suffering from dopaminergic therapy associated psychosis;

administering to the patient an effective amount of an inverse agonist selective for a serotonin receptor; whereby symptoms of dopaminergic therapy associated psychosis is reduced.

33. The method of claim 32, wherein the serotonin receptor is a 5HT_{2A} receptor.

34. The method of claim 32, wherein the serotonin receptor is a 5HT_{2C} receptor.

35. The method of claim 32, wherein the inverse agonist binds to a 5HT_{2A} receptor and a 5HT_{2C} receptor.

36. The method of claim 32, wherein the inverse agonist is the compound of formula (I).

37. The method of claim 36, further comprising an anti-psychotic agent in combination with the compound of formula (I).

38. The method of claim 37, wherein the anti-psychotic agent is selected from the group consisting of chlorpromazine (THORAZINETM), haloperidol (HALDOLTM), molindone (MOBANTM), thioridazine (MELLARILTM), a phenothiazine, a butyrophenone, diphenylbutylpiperidine (pimozide), thioxanthines (flupenthixol), substituted benzamides (sulpiride), sertindole, amisulpride, risperidone, clozapine, olanzapine, ziprasidone, aripiprazole, and their active metabolites (N-desmethyloanzapine, N-desmethyloanzapine, 9-OH-risperidone)).

39. The method of claim 32, wherein the patient suffers from a neurodegenerative disease selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, Spinocerebellar Atrophy, Tourette's Syndrome, Friedrich's Ataxia, Machado-Joseph's disease, Lewy Body Dementia, Dystonia, Progressive Supranuclear Palsy, and Frontotemporal Dementia.

40. A method for treating a neuropsychiatric disease comprising:

identifying a patient suffering from a neuropsychiatric disease; and

administering to the patient an effective amount of an inverse agonist selective for a serotonin receptor.

41. The method of claim 40 wherein the neuropsychiatric disease is selected from the group consisting of schizophrenia, schizoaffective disorders, mania, behavioral disturbances associated with dementia and psychotic depression.

42. The method of claim 40, wherein the serotonin receptor is a 5HT_{2A} receptor.

43. The method of claim 40, wherein the serotonin receptor is a 5HT_{2C} receptor.

44. The method of claim 40, wherein the inverse agonist binds to a 5HT_{2A} receptor or a 5HT_{2C} receptor.

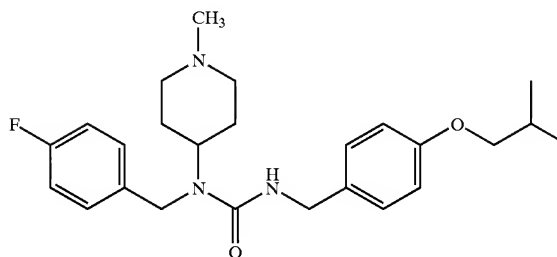
45. The method of claim 40, wherein the inverse agonist is the compound of formula (I).

46. The method of claim 40, further comprising administering an antipsychotic agent in combination with the inverse agonist, the anti-psychotic agent selected from the group consisting of chlorpromazine (THORAZINETM), haloperidol (HALDOLTM), molindone (MOBANTM), thioridazine (MELLARILTM), a phenothiazine, a butyrophenone, diphenylbutylpiperidine (pimozide), thioxanthines (flupenthixol), substituted benzamides (sulpiride), sertindole, amisulpride, risperidone, clozapine, olanzapine, ziprasidone, aripiprazole, and their active metabolites (N-desmethyloanzapine, N-desmethyloanzapine, 9-OH-risperidone)).

47. The method of claim 46, wherein the inverse agonist is the compound of formula (I).

48. A compound having the structure of Formula (I):

(I)



49. A method of inhibiting an activity of a monoamine receptor comprising contacting the monoamine receptor or a system containing the monoamine receptor with an amount of the compound of formula (I) that is effective in inhibiting the activity of the monoamine receptor.

50. The method of claim 49 wherein the monoamine receptor is a serotonin receptor.

51. The method of claim 50 wherein the serotonin receptor is the 5-HT_{2A} subclass.

52. The method of claim 50 wherein the serotonin receptor is in the central nervous system.

53. The method of claim 50 wherein the serotonin receptor is in the peripheral nervous system.

54. The method of claim 50 wherein the serotonin receptor is in blood cells or platelets.

55. The method of claim 50 wherein the serotonin receptor is mutated or modified.

56. The method of claim 49 wherein the activity is signaling activity.

57. The method of claim 49 wherein the activity is constitutive.

58. The method of claim 49 wherein the activity is associated with serotonin receptor activation.

59. A method of inhibiting an activation of a monoamine receptor comprising contacting the monoamine receptor or a system containing the monoamine receptor with an amount of the compound of formula (I) that is effective in inhibiting the activation of the monoamine receptor.

60. The method of claim 59 wherein the activation is by an agonistic agent.

61. The method of claim 60 wherein the agonistic agent is exogenous.

62. The method of claim 60 wherein the agonistic agent is endogenous.

63. The method of claim 59 wherein the activation is constitutive.

64. The method of claim 59 wherein the monoamine receptor is a serotonin receptor.

65. The method of claim 64 wherein the serotonin receptor is the 5-HT_{2A} subclass.

66. The method of claim 64 wherein the serotonin receptor is in the central nervous system.

67. The method of claim 64 wherein the serotonin receptor is in the peripheral nervous system.

68. The method of claim 64 wherein the serotonin receptor is in blood cells or platelets.

69. The method of claim 64 wherein the serotonin receptor is mutated or modified.

70. A method of treating a disease condition associated with a monoamine receptor comprising administering to a subject in need of such treatment a therapeutically effective amount of the compound of formula (I).

71. The method of claim 70 wherein the disease condition is selected from the group consisting of schizophrenia, psychosis, migraine, hypertension, thrombosis, vasospasm, ischemia, depression, anxiety, sleep disorders and appetite disorders.

72. The method of claim 70 wherein the disease condition is associated with dysfunction of a monoamine receptor.

73. The method of claim 70 wherein the disease condition is associated with activation of a monoamine receptor.

74. The method of claim 70 wherein the disease condition is associated with increased activity of monoamine receptor.

75. The method of claim 70 wherein the monoamine receptor is a serotonin receptor.

76. The method of claim 75 wherein the serotonin receptor is the 5-HT_{2A} subclass.

77. The method of claim 75 wherein the serotonin receptor is in the central nervous system.

78. The method of claim 75 wherein the serotonin receptor is in the peripheral nervous system.

79. The method of claim 75 wherein the serotonin receptor is in blood cells or platelets.

80. The method of claim 75 wherein the serotonin receptor is mutated or modified.

81. A method of treating schizophrenia comprising administering to a subject in need of such treatment a therapeutically effective amount the compound of formula (I).

82. A method of treating migraine comprising administering to a subject in need of such treatment a therapeutically effective amount of the compound of formula (I).

83. A method of treating psychosis comprising administering to a subject in need of such treatment a therapeutically effective amount of the compound of formula (I).

84. A method for identifying a genetic polymorphism predisposing a subject to being responsive the compound of formula (I), comprising:

administering to a subject a therapeutically effective amount of said compound; measuring the response of said subject to said compound, thereby identifying a responsive subject having an ameliorated disease condition associated with a monoamine receptor; and

identifying a genetic polymorphism in the responsive subject, wherein the genetic polymorphism predisposes a subject to being responsive to said compound.

85. The method of claim 84 wherein the ameliorated disease condition is associated with the 5-HT class or 5-HT_{2A} subclass of monoaminergic receptors.

86. A method for identifying a subject suitable for treatment with the compound of formula (I), comprising detecting the presence of a polymorphism in a subject wherein the polymorphism predisposes the subject to being responsive to the compound, and wherein the presence of the polymorphism indicates that the subject is suitable for treatment with the compound of formula (I).

* * * * *

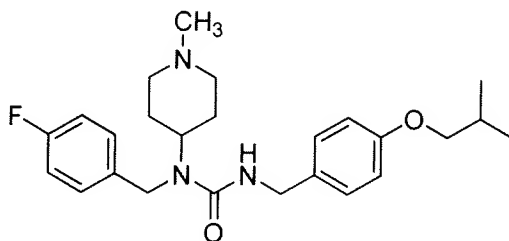
EXHIBIT

3

Exhibit 3

Rejected Claims of U.S. Patent Application No. 10/759,561

1. A composition comprising a compound of Formula (I):



(I)

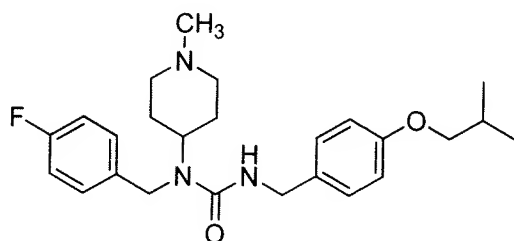
- or the tartrate or hydrochloride salts thereof, and a pharmaceutically acceptable carrier.
2. The composition of claim 1, further comprising an additional therapeutic agent.
3. The composition of claim 2, wherein the additional therapeutic agent is selected from the group consisting of levodopa (SINEMETTM, SINEMET-CRTM, bromocriptine (PARODELTM), pergolide (PERMAXTM), ephedrine sulfate (EPHEDRINETM), pemoline CYLERTTM), mazindol (SANOREXTM), d,1- α -methylphenethylamine (ADDERALLTM), methylphenidate (RITALINTM), pramipexole (MIRAPEXTM), modafinil (PROVIGILTM), and ropinirole (REQUIPTM).
4. The composition of claim 2, wherein the additional therapeutic agent is an anti-dyskinesia agent.
5. The composition of claim 2, wherein the additional therapeutic agent is an anti-dyskinesia agent selected from the group consisting of baclofen (LioresalTM), botulinum toxin (BotoxTM), clonazepam (KlonopinTM), and diazepam (ValiumTM).
6. The composition of claim 2, wherein the additional therapeutic agent is an anti-dystonia, anti-myoclonus, or anti-tremor agent selected from the group consisting of baclofen (LIORESALTM), botulinum toxin (BOTOXTM), clonazepam (KLONOPINTM), and diazepam (VALIUMTM).
7. The composition of claim 2, wherein the additional therapeutic agent is an anti-psychotic agent with dopaminergic receptor antagonism.

Exhibit 3

8. The composition of claim 2, wherein the additional therapeutic agent is an anti-psychotic agent selected from the group consisting of chlorpromazine (THORAZINETM), haloperidol (HALDOLTM), molindone (MOBANTM), thioridazine (MELLARILTM), a phenothiazine, a butyrophenone, diphenylbutylpiperidine (pimozide), thioxanthines (flupenthixol), substituted benzamides (sulpiride), sertindole, amisulpride, ziprasidone, aripiprazole, clozapine, olanzapine, risperidone, and their active metabolites (N-desmethyloclozapine, N-desmethylolanzapine, and 9-OH-resperidone).

9. – 47. Canceled.

48. A compound having the structure of Formula (I):



(I)

or the tartrate or hydrochloride salts thereof.

49. – 89. Withdrawn.

EXHIBIT

4



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Office Action Summary

Application No.

10/759,561

Applicant(s)

WEINER ET AL.

Examiner

Leonard M. Williams

Art Unit

1617

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Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☐ Responsive to communication(s) filed on ____.
- 2a) ☐ This action is FINAL. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-86 is/are pending in the application.
4a) Of the above claim(s) 9-47 and 49-86 is/are withdrawn from consideration.
- 5) ☐ Claim(s) ____ is/are allowed.
- 6) ☒ Claim(s) 1-8 and 48 is/are rejected.
- 7) ☐ Claim(s) ____ is/are objected to.
- 8) ☐ Claim(s) ____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on ____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
a) ☐ All b) ☐ Some * c) ☐ None of:
- ☐ Certified copies of the priority documents have been received.
 - ☐ Certified copies of the priority documents have been received in Application No. ____.
 - ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- ☒ Notice of References Cited (PTO-892)
- ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- ☒ Information Disclosure Statement(s) (PTO/SB/08)
Paper No(s)/Mail Date See Continuation Sheet.
- ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date ____.
- ☐ Notice of Informal Patent Application
- ☐ Other: ____.

Continuation of Attachment(s) 3). Information Disclosure Statement(s) (PTO/SB/08), Paper No(s)/Mail Date :8/8/07, 8/6/07,01/29/07,01/18/05, 5/17/04.

Detailed Action

Election/Restrictions

Applicant's election without traverse of Group I (claims 1-8 and 48) in the reply filed on 08/06/2007 is acknowledged.

Claims 9-47 and 49-86 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on 08/06/2007.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 1-8 and 48 are rejected under 35 U.S.C. 103(a) as being unpatentable over Anderson et al. (WO 0166521-IDS 01/18/2005) and further in view of Goodman and Gilman's The Pharmacological Basis of Therapeutics, 7th edition, pp. 340-343 and 403-404.

Anderson et al. teach, on pages 9-12, compounds of formula 1 that broadly encompass the currently disclosed formula 1. On page 32 lines 12-25, Anderson et al. disclose the co-administration of the compounds of formula 1 with either another compound of formula 1 or another active agent.

On page 6, Anderson et al. disclose that the compounds of formula 1 are useful for treating a variety of diseases and disorders including schizophrenia, depression, anxiety, sleep disorders, etc...

Anderson et al. discloses inverse agonists which are selective for a serotonin receptor. Moreover, Anderson et al. disclose that the inverse agonists of the invention

"avoid the adverse side effects associated with non-selective receptor interactions" (page 4 lines 28-33). Among the side-effects dyskinesia, tremor and dystonic reactions are explicitly mentioned (see page 3 lines 9-10). The therapeutic applications: neurodegenerative diseases, psychosis, schizophrenia, depression and affective disorders are explicitly mentioned to be treatable with ALL compounds of Anderson et al..

Anderson et al. does not disclose in particular drug for use with the compounds of formula 1. The present applications claims 1-3 do not indicate any particular need or reason for any of the additional potential additional therapeutic agents.

Goodman and Gilman's teaches, on pages 340-33, benzodiazepines as useful in the treatment of anxiety, muscle relaxation and anticonvulsive therapy. On page 342, clonazepam is taught as a particularly good muscle relaxant. On page 343, it is disclosed that benzodiazepines increase the net total sleep time, making them suitable as agents for sleep disorders.

Goodman and Gilman's teaches, on pages 403-404, several anti-psychotic agents including thorazine, mellaril, haldol, etc...

It would have been obvious to one of ordinary skill in the art at the time of the invention to utilize the particularly claimed compound of claim 1, as it was disclosed in the genus of formula 1 of Anderson et al. The compounds of both applications are drawn to equivalent compounds and methods of use and as such it is *prima facie*

obvious to utilize the specific compound currently claimed. Further Anderson et al. discloses the coadministration of the compounds of Formula 1 with other active agents.

The examiner respectfully points out the following from MPEP 2144.06:

"It is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose, in order to form a third composition to be used for the very same purpose.... [T]he idea of combining them flows logically from their having been individually taught in the prior art." *In re Kerkhoven*, 626 F.2d 846, 850, 205 USPQ 1069, 1072 (CCPA 1980).

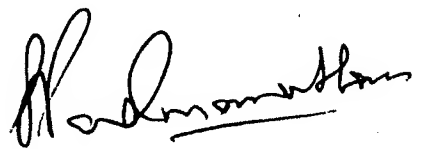
Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Leonard M. Williams whose telephone number is 571-272-0685. The examiner can normally be reached on MF 9-5:30.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sreeni Padmanabhan can be reached on 571-272-0629. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

LMW



SREENI PADMANABHAN
SUPERVISORY PATENT EXAMINER



PTO/SB/08 Equivalent

INFORMATION DISCLOSURE
STATEMENT BY APPLICANT

(Multiple sheets used when necessary)

SHEET 1 OF 10

Application No.	10/759,561
Filing Date	January 15, 2004
First Named Inventor	David M. Weiner, et al.
Art Unit	1617
Examiner	Williams, Leonard M.
Attorney Docket No.	ACADIA.030A

U.S. PATENT DOCUMENTS

Examiner Initials	Cite No.	Document Number Number - Kind Code (if known) Example: 1,234,567 B1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear
/LW/	1	4,138,492	2/6/1979	Noverola, et al.	
	2	4,255,432	3/10/1981	Kluge, et al.	
	3	4,332,804	6/1/1982	Clark	
	4	4,353,900	10/12/1982	Clark	
	5	4,353,901	10/12/1982	Clark	
	6	4,367,232	1/4/1983	Boix-Iglesias, et al.	
	7	5,025,013	6/18/1991	Barreau, et al.	
	8	5,214,055	5/25/1993	Peglion, et al.	
	9	5,461,066	10/24/1995	Gericke, et al.	
	10	5,595,872	1/21/1997	Wetterau II, et al.	
	11	5,621,010	4/15/1997	Sueda, et al.	
	12	5,795,894	8/18/1998	Shue, et al.	
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	14	6,107,324	8/22/2000	Behan, et al.	
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	16	7,022,698	4/4/2006	Hamied, et al.	
	17	7,041,667	5/9/2006	Armour, et al.	
	18	2004/0213816 A1	10/28/2004	Weiner, et al.	
	19	2005/0148018 A1	7/7/2005	Weiner, et al.	
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	24	2006/0205781 A1	9/14/2006	Thygesen, et al.	
	25	2006/0264466 A1	11/23/2006	Weiner, et al.	
	26	2006/0286610 A1	12/21/2006	Brann	
/LW/	27	2006/0292606 A1	12/28/2006	Brann	

Examiner Signature /Leonard Williams/ (09/30/2007)

Date Considered

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T¹ - Place a check mark in this area when an English language Translation is attached.

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	Filing Date	January 15, 2004
	First Named Inventor	David M. Weiner, et al.
	Art Unit	1617
SHEET 2 OF 10	Examiner	Williams, Leonard M.
	Attorney Docket No.	ACADIA.030A

FOREIGN PATENT DOCUMENTS						
Examiner Initials	Cite No.	Foreign Patent Document Country Code-Number-Kind Code Example: JP 1234567 A1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear	T ¹
/LW/	28	CA 984843	3/2/1976	John Wyeth & Brother Limited		
	29	EP 0 005 318 A1	11/14/1979	Janssen Pharm.		
	30	EP 0 061 333 A1	9/29/1982	Syntex (U.S.A.) Inc.		
	31	EP 0 379 441 A1	7/25/1990	Rhone-Poulenc Sante		
	32	EP 0 548 015 A1	6/23/1993	Ciba-Geigy AG		
	33	EP 0 625 507 A2	11/23/1994	Nisshin Flour Milling Co., Ltd.		
	34	HU 157325	3/19/1998	Rudolf, et al.		
	35	WO 97/08168 A1	3/6/1997	Schering Corporation		
	36	WO 97/11940 A1	4/3/1997	Eli Lilly and Company		
	37	WO 98/11128 A1	3/19/1998	Dr. Karl Thomae GMBH		
	38	WO 98/17846 A1	4/30/1998	Dr. Karl Thomae GMBH		
	39	WO 98/44921 A1	10/15/1998	Merck & Co., Inc.		
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	44	WO 01/44191 A1	6/21/2001	Societe de Conseils de Recherches et D'Applications Scientifiques (S.C.R.A.S.)		
	45	WO 01/87839 A1	11/22/2001	Astrazeneca AB		
	46	WO 02/079186 A2	10/10/2002	F. Hoffman-La Roche AG		
	47	WO 03/070246 A1	8/28/2003	Pfizer Products Inc.		
	48	WO 2005/112927 A	12/1/2005	Acadia Pharmaceuticals Inc.		
	49	WO 2006/036874 A1	4/6/2006	Acadia Pharmaceuticals Inc.		
/LW/	50	WO 2006/037043 A1	4/6/2006	Acadia Pharmaceuticals Inc.		

NON PATENT LITERATURE DOCUMENTS			
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/LW/	51	ADAM, et al. 1989. Effects of repeated ritanserine on middle-aged poor sleepers. <i>Psychopharmacology</i> , 99:219-221.	
	52	ADELL, et al. 2005. Strategies for producing faster acting antidepressants. <i>Drug Discovery Today</i> , 10(8):578-585.	
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	64	BIAGI, et al. 1988. 1,2,3-Triazoles: Structural changes on two effective inhibitors of the prostaglandin synthesis <i>in vitro</i> . <i>Farmaco Ed. Sci.</i> , 43:597-612.	
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↓	68	BROWN, et al. 1924. Catalytic alkylation of aniline, <i>J. Am. Chem. Soc.</i> , 46(8):1836-1839.	
/LW/	69	BUCHI et al. 1969. Synthesis of (±)-nuciferal. <i>J. Org. Chem.</i> , 34(4):1122-1123.	

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/LW/	70	BUU-HOI, et al. 1951. Further studies in the alkylation of phenols and thiophenols. <i>J. Org. Chem.</i> , 16:988-994.	
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	86	GAINETDINOV, et al. 2001. Genetic animal models: Focus on schizophrenia. <i>Trends in Neurosciences</i> , 24(9):527-533.	
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/LW/	88	GILLMAN, P. K. 2005. Monoamine oxidase inhibitors, opioid analgesics and serotonin toxicity. <i>British Journal of Anaesthesia</i> , 95(4):434-441.	
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	100	KLAPARS, et al. 2001. A general and efficient copper catalyst for the amidation of aryl halides and the N-arylation of nitrogen heterocycles. <i>J. Am. Chem. Soc.</i> , 123:7727-7729.	
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Multiple sheets used when necessary)	Application No.	10/759,561
	Filing Date	January 15, 2004
	First Named Inventor	David M. Weiner, et al.
	Art Unit	1617
SHEET 6 OF 10	Examiner	Williams, Leonard M.
	Attorney Docket No.	ACADIA.030A

NON PATENT LITERATURE DOCUMENTS			
Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ¹
/LW/	106	KWONG, et al. 2003. Mild and efficient copper-catalyzed amination of aryl bromides and primary alkylamines. <i>Organic Letters</i> , 5(8):793-796.	
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	First Named Inventor	David M. Weiner, et al.
	Art Unit	1617
SHEET 7 OF 10	Examiner	Williams, Leonard M.
	Attorney Docket No.	ACADIA.030A

NON PATENT LITERATURE DOCUMENTS			
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	Filing Date	January 15, 2004
	First Named Inventor	David M. Weiner, et al.
	Art Unit	1617
	Examiner	Williams, Leonard M.
SHEET 9 OF 10	Attorney Docket No.	ACADIA.030A

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/LW/	159	Notice of Allowance and Fee(s) Due and Notice of Allowability dated May 15, 1997, from Application No. 08/273,669 filed July 12, 1994, now U.S. Pat. No. 5,707,798.	
	160	Office Action dated March 27, 1998, from Application No. 08/954,724 filed October 20, 1997, now U.S. Pat. No. 5,912,132.	
	161	Notice of Allowance and Fee(s) Due and Notice of Allowability dated September 4, 1998, from Application No. 08/954,724 filed October 20, 1997, now U.S. Pat. No. 5,912,132.	
	162	Office Action dated September 14, 1998, from Application No. 08/965,947 filed November 7, 1997, now U.S. Pat. No. 5,955,281.	
	163	Interview Summary dated November 17, 1998, from Application No. 08/965,947 filed November 7, 1997, now U.S. Pat. No. 5,955,281.	
	164	International Search Report dated July 17, 2001 for PCT/US01/07187.	
	165	Written Opinion dated November 22, 2002 for PCT/US01/07187.	
	166	Office Action dated February 28, 2001, from Application No. 09/413,626 filed October 6, 1999, now U.S. Pat. No. 6,358,698.	
	167	Notice of Allowance and Fee(s) Due and Notice of Allowability dated November 20, 2001, from Application No. 09/413,626 filed October 6, 1999, now U.S. Pat. No. 6,358,698.	
	168	Office Action dated January 17, 2006, from Application No. 11/154,083 filed June 26, 2005.	
	169	International Search Report dated May 8, 2003 for PCT/US02/41476.	
	170	International Search Report dated January 30, 2006, for PCT/US2005/034813	
	171	Written Opinion of the International Searching Authority dated January 30, 2006, for PCT/US2005/034813.	
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/LW/	177	Office Action dated April 6, 2007, from Application No. 11/418,322 filed May 3, 2006.	

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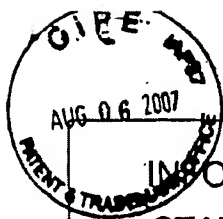
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	Application No.	10/759,561
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/LW/	178	Office Action dated May 8, 2007, from Application No. 11/417,866 filed May 3, 2006.	
/LW/	179	Notice of Allowance and Fee(s) Due and Notice of Allowability dated March 5, 2007, from Application No. 10/601,070 filed June 20, 2003.	
/LW/	180	Office Action dated February 5, 2007; from Application No. 11/299,566 filed December 12, 2005	
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PTO/SB/08 Equivalent

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SHEET 1 OF 1	Attorney Docket No.	ACADIA.030A

U.S. PATENT DOCUMENTS					
Examiner Initials	Cite No.	Document Number Number - Kind Code (if known) Example: 1,234,567 B1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear
/LW/	1	6,150,393	11-21-2000	Behan, et al.	
/LW/	2	6,479,480	11-12-2002	Moyes, et al.	
/LW/	3	6,486,153	11-26-2002	Castro Pineiro, et al.	
/LW/	4	2002/0156068	10-24-2002	Behan et al.	

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Examiner Initials	Cite No.	Foreign Patent Document Country Code-Number-Kind Code Example: JP 1234567 A1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear	T ¹
/LW/	5	WO 2002/076464 A1	10-03-2002	Arena Pharmaceuticals, Inc.		
/LW/	6	WO 2003/062206 A2	07-31-2003	Arena Pharmaceuticals, Inc.		
/LW/	7	WO 2004/039322 A2	05-13-2004	Micro, Inc.		
/LW/	8	WO 2004/064738 A2	08-05-2004	Acadia Pharmaceuticals Inc.		
/LW/	9	WO 2004/064753 A2	08-05-2004	Acadia Pharmaceuticals Inc.		
/LW/	10	WO 2005/053796 A1	06-16-2005	B&B Beheer NV		
/LW/	11	WO 2005/063254 A2	07-14-2005	Acadia Pharmaceuticals Inc.		
/LW/	12	WO 2006/104826	10-05-2006	Merck & Co., Inc.		

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/LW/	13	International Search Report and Written Opinion from PCT/US2005/017808 mailed September 29, 2005.	

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/LW/	1	4,853,394	08-01-1989	King, et al.	
	2	5,216,165	08-01-1993	Mobilio, et al.	
	3	6,756,393	08-29-2004	Andersson, et al.	
	4	6,815,458	11-09-2004	Andersson, et al.	
	5	6,911,452	06-28-2005	Schlienger	
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	7	2004/0006081	01-08-2004	Burrows, et al.	
	8	2004/0106800 A1	06-03-2004	Andersson, et al.	
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	11	2006/0094758 A1	05-04-2006	Andersson, et al.	
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	13	2006/0194834 A1	08-31-2006	Andersson, et al.	
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	15	2006/0199818 A1	09-07-2006	Andersson, et al.	
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	18	2006/0199842 A1	09-07-2006	Weiner, et al.	
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/LW/	20	2006/0264486 A1	11-23-2006	Weiner, et al.	

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/LW/	21	WO 94/27967 A1	12-18-1994	Smithkline Beecham Laboratoires Pharmaceutiques		
/LW/	22	WO 97/38665 A2	10-23-1997	Merck & Co., Inc.		
/LW/	23	WO 97/38984 A1	10-23-1997	The Dupont Merck Pharmaceutical Company		

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/LW/	24	WO 98/50534 A1	11-12-1998	Smithkline Beecham Corporation		
/LW/	25	WO 00/59497 A1	10-12-2000	Merck & Co., Inc.		
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Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ¹
/LW/	34	KALGUTKAR, et al. 1996. Selective inhibitors of monoamine oxidase (MAO-A and MAO-B) as probes of its catalytic site and mechanism. <i>Medicinal Research Reviews</i> , 15(4)325-388. XP002034298.	
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/LW/	36	Office Action dated January 21, 2003, from Application No. 09/800,096, now U.S. Pat. No. 6,815,458.	
/LW/	37	Office Action dated July 15, 2003, from Application No. 09/800,096, now U.S. Pat. No. 6,815,458.	
/LW/	38	Notice of Allowability dated December 8, 2003, from Application No. 09/800,096 filed March 6, 2001, now U.S. Pat. No. 6,815,458.	
/LW/	39	Notice of Allowance and Fee(s) Due and Notice of Allowability dated December 5, 2003, from Application No. 10/409,782 filed April 7, 2003, now U.S. Pat. No. 6,756,393.	
/LW/	40	International Preliminary Examination Report dated March 18, 2003, for PCT/US01/07187.	

Examiner Signature	/Leonard Williams/ (10/01/2007)	Date Considered
*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.		

T¹ - Place a check mark in this area when an English language Translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Multiple sheets used when necessary)</i>	Application No.	10/759,561
	Filing Date	January 15, 2004
	First Named Inventor	Weiner, et al.
	Art Unit	1617
SHEET 3 OF 3		Examiner Williams, Leonard M.
		Attorney Docket No. ACADIA.030A

NON PATENT LITERATURE DOCUMENTS			
Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ¹
/LW/	41	Office Action dated May 21, 2004, from Application No. 10/329,719 filed December 23, 2002, now U.S. Pat. No. 6,911,452.	
/LW/	42	Notice of Allowance and Fee(s) Due and Notice of Allowability dated February 11, 2005, from Application No. 10/329,719 filed December 23, 2002, now U.S. Pat. No. 6,911,452.	
/LW/	43	Office Action dated June 26, 2006, from Application No. 11/154,083 filed June 26, 2005.	
/LW/	44	Notice of Allowability, Notice of Allowance and Fee(s) Due, and Interview Summary dated December 15, 2006, from Application No. 11/154,083 filed June 18, 2005.	
/LW/	45	Office Action dated October 5, 2006, from Application No. 11/418,322 filed May 3, 2006.	
/LW/	46	Office Action dated January 23, 2007, from Application No. 11/418,322 filed May 3, 2006.	
/LW/	47	Written Opinion dated September 9, 2003, for PCT/US02/41476.	
/LW/	48	International Preliminary Examination Report dated January 15, 2004, for PCT/US02/41476.	
/LW/	49	Office Action dated November 4, 2004, from Application No. 10/601,070 filed June 20, 2003.	
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/LW/	51	Notice of Allowance and Fee(s) Due and Notice of Allowability dated March 29, 2006, from Application No. 10/601,070 filed June 20, 2003.	
/LW/	52	International Search Report for PCT/US03/19797 dated December 3, 2003.	
/LW/	53	Written Opinion for PCT/US03/19797 dated April 5, 2004.	
/LW/	54	International Preliminary Examination Report for PCT/US03/19797 dated July 28, 2004.	
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/LW/	56	International Written Opinion for PCT/US2004/001234 dated September 8, 2004.	
/LW/	57	International Preliminary Report on Patentability for PCT/US2004/001234 dated April 14, 2005.	

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PTO/SB/08 Equivalent

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Multiple sheets used when necessary) SHEET 1 OF 1	Application No.	10/759,561
	Filing Date	January 15, 2004
	First Named Inventor	David M. Weiner
	Art Unit	1814
	Examiner	Not Yet Assigned
	Attorney Docket No.	ACADIA.030A

U.S. PATENT DOCUMENTS					
Examiner Initials	Cite No.	Document Number Number - Kind Code (if known) Example: 1,234,567 B1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear
/LW/	1	US 5,877,173 A	03-02-1999	Farber et al.	
/LW/	2	US 3,983,234 A	09-28-1976	Campbell	
/LW/	3	US 2002/165225 A1	11-07-02	Kankan et al.	
/LW/	4	US 6,358,698 B1	03-19-02	Brann et al.	


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Examiner Initials	Cite No.	Foreign Patent Document Country Code-Number-Kind Code Example: JP 1234567 A1	Publication Date MM-DD-YYYY	Name of Patentee or Applicant	Pages, Columns, Lines Where Relevant Passages or Relevant Figures Appear	T ¹
/LW/	5	WO 0166521	09-13-01	Andersson et al.		

NON PATENT LITERATURE DOCUMENTS				
Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ¹	
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/LW/	13	International Search Report mailed on September 8, 2004		
/LW/	14	Written Opinion mailed on December 15, 2004		

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Examiner Signature /Leonard Williams/ (10/01/2007)	Date Considered
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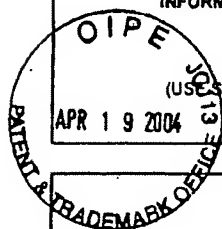
	FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTY. DOCKET NO. ACADIA.030A	APPLICATION NO. 10/759,561
	INFORMATION DISCLOSURE STATEMENT BY APPLICANT		APPLICANT Weiner, et al.	
	USE SEVERAL SHEETS IF NECESSARY)		FILING DATE January 15, 2004	GROUP Unknown

EXAMINER INITIAL	OTHER DOCUMENTS (INCLUDING AUTHOR, TITLE, DATE, PERTINENT PAGES, ETC.)	
/LW/	1	Barchas, et al., <i>Serotonin and Behavior</i> , pp. 483-498, 523-560 (1973)
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*EXAMINER: INITIAL IF CITATION CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP 809; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED, INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.	

FORM PTO-1449	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. ACADIA.030A	APPLICATION NO. 10/759,561
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (USE SEVERAL SHEETS IF NECESSARY)		APPLICANT Weiner, et al.	
		FILING DATE January 15, 2004	GROUP Unknown



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EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE (IF APPROPRIATE)
/LW/	1	5,707,798	01/13/98	Brann			
/LW/	2	5,912,132	06/15/99	Brann			
/LW/	3	5,955,281	09/21/99	Brann			
/LW/	4	2002/0004513	01/10/02	Andersson et al.			
/LW/	5	6,358,698	03/19/02	Weiner et al.			

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FORM PTO-1449	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY. DOCKET NO. ACADIA.030A	APPLICATION NO. 10/759,581
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (USE SEVERAL SHEETS IF NECESSARY)		APPLICANT Weiner, et al.	
		FILING DATE January 15, 2004	GROUP Unknown

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/LW/	37 Weiner, D., M., Burstein, E., S., Nash, N., Croston, G., E., Currier, E., A., Vanover, K., E., Harvey, S., C., Donohue, E., Hansen, H., C., Andersson, C., M., Spalding, T., A., Gibson, D., F., Krebs-Thomson, K., Powell, S., B., Geyer, M., A., Hacksell, U., and Brann, M., R. (2001) "5-Hydroxytryptamine _{2A} Receptor Inverse Agonists as Antipsychotics," <i>J Pharmacol Exp Ther.</i> , 299(1): 268-76.

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EXAMINER /Leonard Williams/ (10/01/2007)	DATE CONSIDERED
*EXAMINER: INITIAL IF CITATION CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP 609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED, INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.	

Notice of References Cited	Application/Control No. 10/759,561	Applicant(s)/Patent Under Reexamination WEINER ET AL.	
	Examiner Leonard M. Williams	Art Unit 1617	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
*	N	WO0166521A1	09-2001	WIPO	ANDERSON ET AL.	
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Goodman and Gilman's The Pharmacological Basis of Therapeutics, 7th edition, pp. 340-343 and 403-404.
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	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707 05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Index of Claims



Application/Control No.

10/759,561

Examiner

Leonard M. Williams

Applicant(s)/Patent under Reexamination

WEINER ET AL.

Art Unit

1617

R	Rejected
=	Allowed

-	(Through numeral) Cancelled
+	Restricted

N	Non-Elected
I	Interference

A	Appeal
O	Objected

Claim		Date						
Final	Original	9/30/07						
	1	R						
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Search Notes

Application/Control No.

10/759,561

Examiner

Leonard M. Williams

Applicant(s)/Patent under
Reexamination

WEINER ET AL.

Art Unit

1617

SEARCHED

Class	Subclass	Date	Examiner
514	310	9/30/2007	LMW

INTERFERENCE SEARCHED

Class	Subclass	Date	Examiner

**SEARCH NOTES
(INCLUDING SEARCH STRATEGY)**

	DATE	EXMR
PALM/EAST INVENTOR NAME	9/30/2007	LMW

EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	21	ritaserin	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2007/10/01 07:54
L2	0	ac90179	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2007/10/01 07:54
L3	0	"ac90179"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2007/10/01 07:54
L4	2	("5707798").PN.	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2007/10/01 07:55
L5	1	("200166521").PN.	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2007/10/01 07:55

EXHIBIT

5



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/759,561	01/15/2004	David M. Weiner	ACADIA.030A	8108

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KNOBBE MARTENS OLSON & BEAR LLP
2040 MAIN STREET
FOURTEENTH FLOOR
IRVINE, CA 92614

EXAMINER	
KIM, JENNIFER M	

ART UNIT	PAPER NUMBER
1617	

NOTIFICATION DATE	DELIVERY MODE
07/11/2008	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

jcartee@kmoh.com
eOAPilot@kmoh.com

Office Action Summary	Application No. 10/759,561	Applicant(s) WEINER ET AL.	
	Examiner Jennifer Kim	Art Unit 1617	

– The MAILING DATE of this communication appears on the cover sheet with the correspondence address –
Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 10 January 2008.
- 2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-8 and 48-89 is/are pending in the application.
- 4a) Of the above claim(s) 49-89 is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 1-8, 48 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. _____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
- * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- | | |
|---|---|
| 1) <input checked="" type="checkbox"/> Notice of References Cited (PTO-892) | 4) <input type="checkbox"/> Interview Summary (PTO-413)
Paper No(s)/Mail Date: _____ |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948) | 5) <input type="checkbox"/> Notice of Informal Patent Application |
| 3) <input type="checkbox"/> Information Disclosure Statement(s) (PTO/SB/08)
Paper No(s)/Mail Date: _____ | 6) <input type="checkbox"/> Other: _____ |

DETAILED ACTION

The amendment filed January 10, 2008 have been received and entered into the application. Newly added claims 87-89 are withdrawn from consideration since they read on the non-elected invention.

Applicant's arguments with respect to claims 1-8 and 48 have been considered but are moot in view of the new ground(s) of rejection.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless —

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claim 48 is rejected under 35 U.S.C. 102(b) as being anticipated by R&D Focus Drug News (12 Nov. 2001).

R&D Focus Drug News teaches the compound set forth in claim 48, ACP 103, is a selective 5HT_{2A} inverse agonist and has a potential antipsychotic agent with an improved side-effect profile. R&D Focus Drug News teaches that ACP 103 was found to be orally bioavailable with a high efficacy, in animal models of psychosis.

Claim 48 is rejected under 35 U.S.C. 102(b) as being anticipated by R&D Focus Drug News (24 Jan 2000).

R&D Focus Drug News teaches pimavanserin tartrate (ACP 103) has been identified as a lead compound within its program to develop as an antipsychotic drug.

Claim Rejections - 35 USC § 103

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claims 1-8 are rejected under 35 U.S.C. 103(a) as being unpatentable over R&D Focus Drug News (12 Nov. 2001) in view of Anderson et al. (WO0166521-IDS 01/18/2005) of record and further in view of and further in view of Goodman and Gilman's The Pharmacological Basis of Therapeutics, 7th edition, pages 340-343 and 403-404 of record.

R&D Focus Drug News teaches the compound set forth in claim 1, ACP 103, is a selective 5HT_{2A} inverse agonist and has a potential antipsychotic agent with an improved side-effect profile. R&D Focus Drug News teaches that ACP 103 was found to be orally bioavailable with a high efficacy, in animal models of psychosis.

R&D Focus Drug News does not expressly teach a pharmaceutically acceptable carrier and an additional therapeutic agent and the additional agents set forth in claims 3-8.

Anderson et al. teach that the compounds broadly including, ACP 103 can be formulated with pharmaceutically acceptable insert carrier such as ethanol, glycerol, water and the like. Anderson et al. teach that when desired or necessary, suitable binders, lubricants, disintegrating agents, flavoring agents and disintegrating agents can be also incorporated into the mixture comprising the compound. (page 29, lines 27-30). Anderson et al. teach on pages 9-12, compounds of formula I that broadly encompasses the currently disclosed formula I and suitable pharmaceutically acceptable salts such including hydrochloride and tartrate. (Page 27, line30-page 28 line 14). On page 32 lines 12-25, Anderson et al. disclose the co-administration of the compounds of formula I with either another compound of formula I or another active agent. One page 6, Anderson et al. disclose that the compounds of formula I are useful for treating variety of diseases and disorders including schizophrenia, depression, anxiety, sleep disorder, etc. Anderson et al. disclose that the formula I, avoid the adverse side effects such as dyskinesia, tremor and dystonic reactions. (page 4, lines 28-33, page 3, lines 9-10). The therapeutic applications: neurodegenerative disease, psychosis, schizophrenia, depression and affective disorders are explicitly mentioned to be treatable with compounds of Anderson et al.

Goodman and Gilman's teaches, on page 340-343 teach, benzodiazepines useful in the treatment of anxiety, muscle relaxation and anticonvulsive therapy. On page 342, clonazepam is taught as a particularly good muscle relaxant. On page 343, it is disclosed that benzodiazepines increase the net total sleep time, making them

unsuitable as agents for sleep disorders. Goodman and Gilman's teaches, on page 403-404, several anti-psychotic agents including Thorazine, Mellaril, Haldol, etc.

It would have been obvious to one of ordinary skill in the art to modify the teaching of R&D Focus Drug News and formulate a pharmaceutical composition comprising ACP 103 by combining a pharmaceutically acceptable insert carriers for treatment of psychosis because R&D Focus Drug News teaches the compound, ACP 103, was found to be orally bioavailable with a high efficacy, in animal models of psychosis and because Anderson teaches that the general compounds including ACP 103 can be combined with a pharmaceutically acceptable carrier for oral administration. One would have been motivated to make such a modification in order to achieve an expected antipsychotic benefit of the ACP 103 in oral formulation with a benefit of having an improved side-effect profile. There is a reasonable expectation of successfully formulating ACP103 with a pharmaceutically acceptable carrier as an antipsychotic agent because there is a clear teaching from R&D Focus Drug News that ACP 103 was found to be orally bioavailable with a high efficacy, in animal models of psychosis. With respect to incorporation of additional agents set forth in claim 3-8 are obvious because Anderson et al. disclose that coadministration of the compounds of formula I with other active agents and that the compound formula I is useful for the treatment of psychosis and related disorders such as neurodegenerative disease, psychosis, schizophrenia, depression and affective disorders and dyskinesia, tremor and dystonic reactions as taught by Anderson et al. It is noted that Goodman and Gilman teaches the additional agents are also useful for the treatment of various

conditions including psychosis, anxiety, sleep disorder and conditions related to psychosis. The motivation for combining the components flows from their individually known common utility (see *In re Kerkhoven*, 205 USPQ 1069(CCPA 1980)). Thus, the claims fail to patentably distinguish over the state of the art as represented by the cited references.

Inventorship

In view of the papers filed June 20, 2008, it has been found that this nonprovisional application, as filed, through error and without deceptive intent, improperly set forth the inventorship, and accordingly, this application has been corrected in compliance with 37 CFR 1.48(a).

The inventorship of this application has been changed by addition of:

Carl-Magnus A. Andersson
Ferievagen 3
SE-245 64 Hjarup, Sweden

and

Allan K. Uldam
Skotteparken 172
2750 Ballerup, Denmark.

The application will be forwarded to the Office of Initial Patent Examination (OIPE) for issuance of a corrected filing receipt, and correction of Office records to reflect the inventorship as corrected.

Communication

Applicants' amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire **THREE MONTHS** from the mailing date of this action. In the event a first reply is filed within **TWO MONTHS** of the mailing date of this final action and the advisory action is not mailed until after the end of the **THREE-MONTH** shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than **SIX MONTHS** from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jennifer Kim whose telephone number is 571-272-0628. The examiner can normally be reached on Monday through Friday 6:30 am to 3 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sreenivasan Padmanabhan can be reached on 571-272-0629. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300. Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Jennifer Kim/
Primary Examiner, Art Unit 1617

Jmk
July 7, 2008

Notice of References Cited	Application/Control No. 10/759,561	Applicant(s)/Patent Under Reexamination WEINER ET AL.	
	Examiner Jennifer Kim	Art Unit 1617	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			


FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS


*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	R&D Focus Drug News (12 Nov 2001). pimvaserin ACADIA preclinical data.
	V	R&D Focus Drug News (24 Jan 2000). pimvanaserin ACADIA lead compounds indentified.
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Index of Claims 	Application/Control No. 10759561	Applicant(s)/Patent Under Reexamination WEINER ET AL.
	Examiner Jennifer Kim	Art Unit 1617


✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

<input type="checkbox"/> Claims renumbered in the same order as presented by applicant			<input type="checkbox"/> CPA			<input type="checkbox"/> T.D.			<input type="checkbox"/> R.1.47		
CLAIM		DATE									
Final	Original	07/07/2008									
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	2	✓									
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Index of Claims 	Application/Control No. 10759561	Applicant(s)/Patent Under Reexamination WEINER ET AL.
	Examiner Jennifer Kim	Art Unit 1617


✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
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CLAIM			DATE					
Final	Original	07/07/2008						
	37	-						
	38	-						
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	47	-						
	48	✓						
	49	N						
	50	N						
	51	N						
	52	N						
	53	N						
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	72	N						

Index of Claims 	Application/Control No. 10759561	Applicant(s)/Patent Under Reexamination WEINER ET AL.
	Examiner Jennifer Kim	Art Unit 1617

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
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<input type="checkbox"/> Claims renumbered in the same order as presented by applicant			<input type="checkbox"/> CPA			<input type="checkbox"/> T.D.			<input type="checkbox"/> R.1.47		
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	83	N									
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	85	N									
	86	N									
	87	N									
	88	N									
	89	N									

Search Notes 	Application/Control No. 10759561	Applicant(s)/Patent Under Reexamination WEINER ET AL.
	Examiner Jennifer Kim	Art Unit 1617

SEARCHED			
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514	310	7/7/2008	jmk

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EXHIBIT

6

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(DK). ULDAM, A., K. [DK/DK]; Raevohjparken 4,2 tv,
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(21) International Application Number: PCT/US01/07187

(74) Agents: **BABIN, Jane, K.** et al.; Pillsbury Winthrop LLP,
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Filed on 6 March 2000 (06.03.2000)

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IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT,
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CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

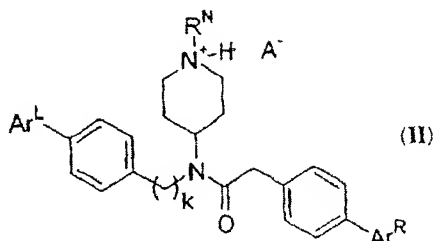
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ning of each regular issue of the PCT Gazette.

WO 01/66521 A1

(54) Title: **AZACYCLIC COMPOUNDS FOR USE IN THE TREATMENT OF SEROTONIN RELATED DISEASES**



(57) **Abstract:** Compound and methods are provided for the treatment of
disease conditions in which modification of serotonergic receptor activity
has a beneficial effect. In the method, an effective amount of a compound
of formula (II) is administered to a patient in need of such treatment.

Field of the Invention

AZACYCLIC COMPOUNDS FOR USE IN THE TREATMENT OF SEROTONIN RELATED DISEASES

5 The present invention relates to novel compounds that affect monoamine receptors, including serotonin receptors. The invention specifically provides compounds that are active as inverse agonists, and therefore also as antagonists, at the 5-HT_{2A} subtype of human serotonin receptors. The invention also provides methods, utilizing the compounds of the invention for modulating 5-HT_{2A} receptor-mediated
10 events, that are useful for treating or alleviating disease conditions in which modification of the activity of these receptors is beneficial.

Background of the Invention

 Serotonin or 5-hydroxytryptamine (5-HT) plays a significant role in the functioning of the mammalian body. In the central nervous system, 5-HT is an
15 important neurotransmitter and neuromodulator that is implicated in such diverse behaviors and responses as sleeping, eating, locomotion, perceiving pain, learning and memory, sexual behavior, controlling body temperature and blood pressure. In the spinal column, serotonin plays an important role in the control systems of the afferent peripheral nociceptors (Moulinier, *Rev. Neurol.* 150:3-15, (1994)). Peripheral
20 functions in the cardiovascular, hematological and gastrointestinal systems have also been ascribed to 5-HT. 5-HT has been found to mediate a variety of contractile, secretory, and electrophysiologic effects including vascular and nonvascular smooth muscle contraction, and platelet aggregation. (Fuller, *Biology of Serotonergic Transmission*, 1982; Boullin, *Serotonin In Mental Abnormalities* 1:316 (1978);
25 Barchas, et al., *Serotonin and Behavior*, (1973)). The 5-HT_{2A} receptor subtype (also referred to as subclass) is widely yet discretely expressed in the human brain, including many cortical, limbic, and forebrain regions postulated to be involved in the modulation of higher cognitive and affective functions. This receptor subtype is also expressed on mature platelets where it mediates, in part, platelet aggregation, one of
30 the initial steps in the process of vascular thrombosis.

 Given the broad distribution of serotonin within the body, it is understandable that tremendous interest in drugs that affect serotonergic systems exists (Gershon, et

al., *The Peripheral Actions of 5-Hydroxytryptamine*, 246 (1989); Saxena, et al., *J. Cardiovascular Pharmacol.* 15: Supp. 7 (1990)). Serotonin receptors are members of a large human gene family of membrane-spanning proteins that function as transducers of intercellular communication. They exist on the surface of various cell types, including neurons and platelets, where, upon their activation by either their endogenous ligand serotonin or exogenously administered drugs, they change their conformational structure and subsequently interact with downstream mediators of cellular signaling. Many of these receptors, including the 5-HT_{2A} subclass, are G-protein coupled receptors (GPCRs) that signal by activating guanine nucleotide binding proteins (G-proteins), resulting in the generation, or inhibition of, second messenger molecules such as cyclic AMP, inositol phosphates, and diacylglycerol. These second messengers then modulate the function of a variety of intracellular enzymes, including kinases and ion channels, which ultimately affect cellular excitability and function.

At least 15 genetically distinct 5-HT receptor subtypes have been identified and assigned to one of seven families (5-HT₁₋₇). Each subtype displays a unique distribution, preference for various ligands, and functional correlate(s).

Serotonin may be an important component in various types of pathological conditions such as certain psychiatric disorders (depression, aggressiveness, panic attacks, obsessive compulsive disorders, psychosis, schizophrenia, suicidal tendency), certain neurodegenerative disorders (Alzheimer-type dementia, Parkinsonism, Huntington's chorea), anorexia, bulimia, disorders associated with alcoholism, cerebral vascular accidents, and migraine (Meltzer, *Neuropsychopharmacology*, 21:106S-115S (1999); Barnes & Sharp, *Neuropharmacology*, 38:1083-1152 (1999); Glennon, *Neurosci. Biobehavioral Rev.*, 14:35 (1990)). Recent evidence strongly implicates the 5-HT₂ receptor subtype in the etiology of such medical conditions as hypertension, thrombosis, migraine, vasospasm, ischemia, depression, anxiety, psychosis, schizophrenia, sleep disorders and appetite disorders.

Schizophrenia is a particularly devastating neuropsychiatric disorder that affects approximately 1% of the human population. It has been estimated that the total financial cost for the diagnosis, treatment, and lost societal productivity of individuals affected by this disease exceeds 2% of the gross national product (GNP) of the United States. Current treatment primarily involves pharmacotherapy with a class of drugs known as antipsychotics. Antipsychotics are effective in ameliorating

positive symptoms (e.g., hallucinations and delusions), yet they frequently do not improve negative symptoms (e.g., social and emotional withdrawal, apathy, and poverty of speech).

Currently, nine major classes of antipsychotics are prescribed to treat
5 psychotic symptoms. Use of these compounds is limited, however, by their side effect profiles. Nearly all of the "typical" or older generation compounds have significant adverse effects on human motor function. These "extrapyramidal" side effects, so termed due to their effects on modulatory human motor systems, can be both acute (e.g., dystonic reactions, a potentially life threatening but rare neuroleptic
10 malignant syndrome) and chronic (e.g., akathisia, tremors, and tardive dyskinesia). Drug development efforts have, therefore, focused on newer "atypical" agents free of these adverse effects.

Antipsychotic drugs have been shown to interact with a large number of central monoaminergic neurotransmitter receptors, including dopaminergic,
15 serotonergic, adrenergic, muscarinic, and histaminergic receptors. It is likely that the therapeutic and adverse effects of these drugs are mediated by distinct receptor subtypes. The high degree of genetic and pharmacological homology between these receptor subtypes has hampered the development of subtype-selective compounds, as well as the determination of the normal physiologic or pathophysiologic role of any
20 particular receptor subtype. Thus there is a need to develop drugs that are selective for individual receptor classes and subclasses amongst monoaminergic neurotransmitter receptors.

The prevailing theory for the mechanism of action of antipsychotic drugs involves antagonism of dopamine D2 receptors. Unfortunately, it is likely that
25 antagonism of dopamine D2 receptors also mediates the extrapyramidal side effects. Antagonism of 5-HT_{2A} is an alternate molecular mechanism for drugs with antipsychotic efficacy, possibly through antagonism of heightened or exaggerated signal transduction through serotonergic systems. 5-HT_{2A} antagonists are therefore good candidates for treating psychosis without extrapyramidal side effects.

30 Traditionally, these receptors have been assumed to exist in a quiescent state unless activated by the binding of an agonist (a drug that activates a receptor). It is now appreciated that many, if not most, of the GPCR monoamine receptors, including serotonin receptors, can exist in a partially activated state in the absence of their endogenous agonists. This increased basal activity (constitutive activity) can be

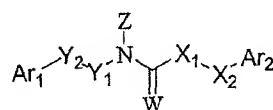
inhibited by compounds called inverse agonists. Both agonists and inverse agonists possess intrinsic activity at a receptor, in that they alone can activate or inactivate these molecules, respectively. In contrast, classic or neutral antagonists compete against agonists and inverse agonists for access to the receptor, but do not possess the
5 intrinsic ability to inhibit elevated basal or constitutive receptor responses.

We have recently elucidated an important aspect of 5-HT_{2A} receptor function by applying the Receptor Selection and Amplification Technology (U.S. Patent 5,707,798, 1998; *Chem Abstr.* 128:111548 (1998) and citations therein), to the study of the 5-HT₂ subclass of serotonin receptors. R-SAT is a phenotypic assay of
10 receptor function that involves the heterologous expression of receptors in mammalian fibroblasts. Using this technology we were able to demonstrate that native 5-HT_{2A} receptors possess significant constitutive, or agonist-independent, receptor activity (U.S. Patent Application Ser. No. 60/103,317, herein incorporated by reference). Furthermore, by directly testing a large number of centrally acting
15 medicinal compounds with known clinical activity in neuropsychiatric disease, we determined that compounds with antipsychotic efficacy all shared a common molecular property. Nearly all of these compounds, which are used by psychiatrists to treat psychosis, were found to be potent 5-HT_{2A} inverse agonists. This unique clinico-pharmacologic correlation at a single receptor subtype is compelling evidence
20 that 5-HT_{2A} receptor inverse agonism is a molecular mechanism of antipsychotic efficacy in humans.

Detailed pharmacological characterization of a large number of antipsychotic compounds revealed that they possess broad activity at multiple related receptor subtypes. Most of these compounds display agonist, competitive antagonist, or
25 inverse agonist activity at multiple monoaminergic receptor subtypes, including serotonergic, dopaminergic, adrenergic, muscarinic and histaminergic receptors. This broad activity is likely responsible for the sedating, hypotensive, and motor side effects of these compounds. It would therefore be of great advantage to develop compounds that are selective inverse agonists of the 5-HT_{2A} receptor, but which have
30 little or no activity on other monamine receptors subtypes, especially dopamine D₂ receptors. Such compounds may be useful in the treatment of human disease (e.g., as anti-psychotics), and may avoid the adverse side effects associated with non-selective receptor interactions.

Summary of the Invention

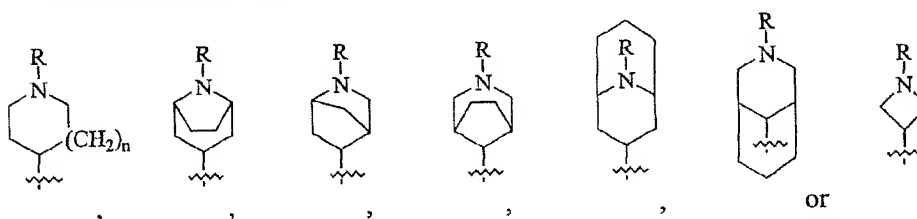
The present invention provides compounds of the general formula (I) that affect monoamine receptors, especially serotonin receptors, and share as a common property inverse agonist activity at the 5-HT_{2A} subtype of human serotonin receptors:



I

5 wherein

Z is a group selected from



10 R is hydrogen, a cyclic or straight-chained or branched acyclic organyl group, a lower hydroxyalkyl group, a lower aminoalkyl group, or an aralkyl or heteroaralkyl group;

n is 0, 1, or 2;

X₁ is methylene, vinylene, or an NH or N(lower alkyl) group; and

15 X₂ is methylene, or, when X₁ is methylene or vinylene, X₂ is methylene or a bond; or when X₁ is methylene, X₂ is O, S, NH, or N(lower alkyl) or a bond;

Y₁ is methylene and Y₂ is methylene, vinylene, ethylene, propylene, or a bond;

or

Y₁ is a bond and Y₂ is vinylene; or

Y₁ is ethylene and Y₂ is O, S, NH, or N(lower alkyl);

20 Ar₁ and Ar₂ independently are unsubstituted or substituted aryl or heteroaryl groups;

W is oxygen or sulfur; or

a pharmaceutically acceptable salt, ester, or prodrug thereof.

25 The present invention also provides pharmaceutical compositions comprising an effective amount of a compound of formula (I) or pharmaceutically acceptable salts, esters, or prodrugs thereof.

Also provided are methods of inhibiting an activity of a monoamine receptor comprising contacting the monoamine receptor or a system containing the monoamine receptor with an effective amount of a compound of formula (I), as well as kits for performing the same. Preferably, the receptor is a serotonin receptor of the 5-HT_{2A} subclass. The receptor may be located in either the central or peripheral nervous system, blood cells or platelets, and may be mutated or modified. In a preferred embodiment, the receptor is constitutively active.

Furthermore, the present invention relates to a method of inhibiting an activation of a monoamine receptor comprising contacting the monoamine receptor or a system containing the monoamine receptor with an effective amount of compound of formula (I), as well as kits for performing the same. In a preferred embodiment, the compound is selective for the 5-HT_{2A} serotonin receptor. In another preferred embodiment, the compound has little or substantially no anti-dopaminergic activity. The receptor may be constitutively active or may be activated by an endogenous or exogenous agonistic agent.

Another aspect of the present invention relates to a method of treating a disease condition associated with a monoamine receptor comprising administering to a mammal in need of such treatment an effective amount of a compound of formula (I), and kits for performing the same. Examples of disease conditions for which such treatment using the compounds of the invention, or pharmaceutical compositions comprising them, is useful include, but are not limited to, neuropsychiatric diseases such as schizophrenia and related idiopathic psychoses, depression, anxiety, sleep disorders, appetite disorders, affective disorders such as major depression, bipolar disorder, and depression with psychotic features, and Tourette's Syndrome. Said compounds may also be beneficial for the treatment of drug-induced psychoses as well as psychoses secondary to neurodegenerative disorders such as Alzheimer's or Huntington's Disease. The compounds of the invention may also be useful in treating hypertension, migraine, vasospasm, ischemia and the primary treatment and secondary prevention of various thrombotic conditions including myocardial infarction, thrombotic or ischemic stroke, idiopathic and thrombotic thrombocytopenic purpura, and peripheral vascular disease.

Further provided is a method for identifying a genetic polymorphism predisposing a subject to being responsive to a compound of formula (I), comprising administering to a subject an effective amount of the compound; identifying a

responsive subject having an ameliorated disease condition associated with a monoamine receptor; and identifying a genetic polymorphism in the responsive subject, wherein the genetic polymorphism predisposes a subject to being responsive to the compound. Also provided are kits for performing the same.

5 A method for identifying a subject suitable for treatment with the compound of formula (I) and kits for identifying the same, is also provided. According to the method, the presence of a polymorphism that predisposes the subject to being responsive to the compound is detected, wherein the presence of the polymorphism indicates that the subject is suitable for treatment.

10 Brief Description of the Drawings

Figure 1 is a graph showing data obtained from a dose response analysis of 26HCH17 and ritanserin as 5-HT_{2A} receptor inverse agonists.

Figure 2 is a graphic representation of *in vivo* pharmacology data obtained in mice with 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)acetamide hydrochloride. Figure 2A shows the effects of this novel antipsychotic agent compound in a head twitch behavioral model; Figure 2B shows the results of locomotor experiments; and in Figure 2C pre-pulse inhibition study results are shown.

Detailed Description of the Invention

Definitions

20 For the purpose of the current disclosure, the following definitions shall in their entireties be used to define technical terms, and shall also, in their entireties, be used to define the scope of the composition of matter for which protection is sought in the claims.

“Constitutive activity” is defined as the elevated basal activity of a receptor which is independent of the presence of an agonist. Constitutive activity of a receptor may be measured using a number of different methods, including cellular (e.g., membrane) preparations (see, e.g., Barr & Manning, *J. Biol. Chem.* 272:32979-87 (1997)), purified reconstituted receptors with or without the associated G-protein in phospholipid vesicles (Cerione et al., *Biochemistry* 23:4519-25 (1984)), and functional cellular assays (U.S. Patent Application Ser. No. 60/103,317).

30 “Agonist” is defined as a compound that increases the activity of a receptor when it contacts the receptor.

An "antagonist" is defined as a compound that competes with an agonist or inverse agonist for binding to a receptor, thereby blocking the action of an agonist or inverse agonist on the receptor. However, an antagonist (also known as a "neutral" antagonist) has no effect on constitutive receptor activity.

5 An "inverse agonist" is defined as a compound that decreases the basal activity of a receptor (i.e., signalling mediated by the receptor). Such compounds are also known as negative antagonists. An inverse agonist is a ligand for a receptor that causes the receptor to adopt an inactive state relative to a basal state occurring in the absence of any ligand. Thus, while an antagonist can inhibit the activity of an agonist,
10 an inverse agonist is a ligand that can alter the conformation of the receptor in the absence of an agonist. The concept of an inverse agonist has been explored by Bond et al. in *Nature* 374:272 (1995). More specifically, Bond et al. have proposed that unliganded β_2 -adrenoceptor exists in an equilibrium between an inactive conformation and a spontaneously active conformation. Agonists are proposed to
15 stabilize the receptor in an active conformation. Conversely, inverse agonists are believed to stabilize an inactive receptor conformation. Thus, while an antagonist manifests its activity by virtue of inhibiting an agonist, an inverse agonist can additionally manifest its activity in the absence of an agonist by inhibiting the spontaneous conversion of an unliganded receptor to an active conformation.

20 The "5-HT_{2A} receptor" is defined as a receptor, having an activity corresponding to the activity of the human serotonin receptor subtype, which was characterized through molecular cloning and pharmacology as detailed in Saltzman et al., *Biochem. Biophys. Res. Comm.* 181:1469-78; and Julius et al., *Proc. Natl. Acad. Sci. USA* 87:928-932.

25 The term "subject" refers to an animal, preferably a mammal, most preferably a human, who is the object of treatment, observation or experiment.

 "Selective" is defined as a property of a compound whereby an amount of the compound sufficient to effect a desired response from a particular receptor type, subtype, class or subclass with substantially little or no effect upon the activity other
30 receptor types. "Selectivity" or "selective," as an inverse agonist is understood as a property of a compound of the invention whereby an amount of compound that effectively inversely agonises the 5-HT_{2A} receptor, and thereby decreases its activity, causes little or no inverse agonistic or antagonistic activity at other, related or

unrelated, receptors. In particular, the compounds of the invention have surprisingly been found not to interact strongly with other serotonin receptors (5-HT 1A, 1B, 1D, 1E, 1F, 2B, 2C, 4A, 6, and 7) at concentrations where the signalling of the 5-HT_{2A} receptor is strongly or completely inhibited. Preferably, the compounds of the invention are also selective with respect to other monoamine-binding receptors, such as the dopaminergic, histaminergic, adrenergic and muscarinic receptors. Compounds that are highly selective for 5-HT_{2A} receptors may have a beneficial effect in the treatment of psychosis, schizophrenia or similar neuropsychiatric disorders, while avoiding adverse effects associated with drugs hitherto suggested for this purpose.

EC₅₀ for an agonist is intended to denote the concentration of a compound needed to achieve 50% of a maximal response seen in R-SAT. For inverse agonists, EC₅₀ is intended to denote the concentration of a compound needed to achieve 50% inhibition of an R-SAT response from basal, no compound, levels.

As used herein, "coadministration" of pharmacologically active compounds refers to the delivery of two or more separate chemical entities, whether in vitro or in vivo. Coadministration refers to the simultaneous delivery of separate agents; to the simultaneous delivery of a mixture of agents; as well as to the delivery of one agent followed by delivery of a second agent or additional agents. In all cases, agents that are coadministered are intended to work in conjunction with each other.

"Cyclic organyl groups" are aliphatic, alicyclic groups in which carbon atoms form a ring. In preferred embodiments containing four, five, six or seven carbon atoms, the ring, as a substituent, is connected either directly via one of the ring atoms or via one or more appended carbon atoms. Particular examples of such groups include cyclopentyl, cyclohexyl, cycloheptyl, cyclopentylmethyl, cyclohexylmethyl, cyclohexylethyl groups, and the like.

"Straight-chained acyclic organyl groups" are substituent groups consisting of a linear arrangement of carbon atoms, where accordingly each carbon atom binds a maximum of two other carbon atoms, connected through single, double, or triple bonds. The straight-chained organyl groups may contain none, one, or several multiple bonds, and are, for example, commonly referred to as alkyl, alkenyl or alkynyl, or alkadienyl groups, respectively. Examples of straight-chained organyl groups include methyl, ethyl, propyl, butyl, pentyl, hexyl, propenyl, butenyl, pentadienyl, propargyl, butynyl.

“Branched acyclic organyl groups” are substituent groups consisting of a branched arrangement of carbon atoms, where accordingly one or more carbon atoms may bind more than two other carbon atoms, connected through single, double, or triple bonds. The branched organyl groups may contain none, one, or several multiple
5 bonds. Examples of branched organyl groups include *iso*-propyl, *iso*-butyl, *tert*-butyl, methylbutyl, methylbutenyl, methylbutynyl.

“Lower alkoxy groups” are C₁₋₆ cyclic or acyclic organyl groups connected, as substituents, via an oxygen atom. Examples of lower alkoxy groups include methoxy, ethoxy, *iso*-propoxy, butoxy, *tert*-butoxy.

10 “Lower alkyl groups” are C₁₋₆ cyclic, straight-chained or branched aliphatic substituent groups connected via a carbon atom. Examples include methyl, ethyl, propyl, butyl, methylbutyl, cyclopropyl, cyclohexyl, *iso*-propyl, *tert*-butyl.

“Lower alkylamino groups” are understood as lower alkyl groups connected, as substituents, via a nitrogen atom, which may carry one or two lower alkyl groups.
15 Particular examples include methylamino, dimethylamino, *iso*-propylamino. Optionally, lower aminoalkyl groups may consist of 4-6 membered nitrogen-containing rings, such as pyrrolidino.

“Lower aminoalkyl groups” are lower alkyl groups carrying, as a substituent, an additional amino group. Examples include aminomethyl and aminoethyl.

20 “Lower hydroxyalkyl groups” are understood as lower alkyl groups carrying, as a substituent, an additional hydroxy group. Examples include hydroxymethyl, hydroxyethyl, 2-hydroxy-2-propyl, hydroxypentyl.

“Acyl groups” are hydrogen or lower alkyl groups connected, as substituents, via a carbonyl group. Examples include formyl, acetyl, propanoyl.

25 “Halo groups” are understood to be fluoro, chloro, bromo, or iodo substituents, with fluoro and chloro being generally preferred.

“Lower alkylene groups” are straight-chained tethering groups, forming bonds to connect molecular fragments via their terminal carbon atoms. Examples include methylene (-CH₂-), ethylene (-CH₂CH₂-), propylene (-CH₂CH₂CH₂-) or butylene
30 (-(CH₂)₄-) groups.

“Vinylene groups” are ethene-1,2-diyl groups (-CHCH-) having (*E*) or (*Z*) configuration.

“Aralkyl groups” are aryl groups connected, as substituents, via a lower alkylene group. The aryl groups of aralkyl groups may be substituted or unsubstituted.

Examples include benzyl, substituted benzyl, 2-phenylethyl, 3-phenylpropyl, naphthylalkyl.

'Heteroaralkyl groups' are understood as heteroaryl groups connected, as substituents, via a lower alkylene group. The heteroaryl groups of heteroaralkyl groups may be substituted or unsubstituted. Examples include 2-thienylmethyl, 3-thienylmethyl, furylmethyl, thienylethyl, pyrrolylalkyl, pyridylalkyl, isoxazolylalkyl, imidazolylalkyl, and their substituted as well as benzo-fused analogs.

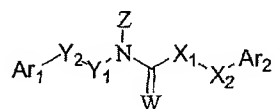
"Aryl groups" are aromatic, preferably benzenoid or naphthoid, groups connected via one of the ring-forming carbon atoms, and optionally carrying one or more substituents selected from halo, hydroxy, amino, cyano, nitro, alkylamido, acyl, lower alkoxy, lower alkyl, lower hydroxyalkyl, lower aminoalkyl, lower alkylamino, alkylsulfenyl, alkylsulfinyl, alkylsulfonyl, sulfamoyl, or trifluoromethyl. Preferred aryl groups are phenyl, and, most suitably, substituted phenyl groups, carrying one or two, same or different, of the substituents listed above. The preferred pattern of substitution is *para* and/or *meta*. Representative examples of aryl groups include, but are not limited to, phenyl, 3-halophenyl, 4-halophenyl, 3-hydroxyphenyl, 4-hydroxyphenyl, 3-aminophenyl, 4-aminophenyl, 3-methylphenyl, 4-methylphenyl, 3-methoxyphenyl, 4-methoxyphenyl, 3-cyanophenyl, 4-cyanophenyl, dimethylphenyl, naphthyl, hydroxynaphthyl, hydroxymethylphenyl, trifluoromethylphenyl.

"Heteroaryl groups" are understood as aromatic, C₂₋₆ cyclic groups containing one O or S atom or up to four N atoms, or a combination of one O or S atom with up to two N atoms, and their substituted as well as benzo- and pyrido-fused derivatives, preferably connected via one of the ring-forming carbon atoms. Heteroaryl groups may carry one or more substituents, selected from halo, hydroxy, amino, cyano, nitro, alkylamido, acyl, lower alkoxy, lower alkyl, lower hydroxyalkyl, lower aminoalkyl, lower alkylamino, alkylsulfenyl, alkylsulfinyl, alkylsulfonyl, sulfamoyl, or trifluoromethyl. Preferred heteroaryl groups are five- and six-membered aromatic heterocyclic systems carrying 0, 1, or 2 substituents, which may be the same as or different from one another, selected from the list above. Representative examples of heteroaryl groups include, but are not limited to, unsubstituted and mono- or di-substituted derivatives of furan, benzofuran, thiophene, benzothiophene, pyrrole, indole, oxazole, benzoxazole, isoxazole, benzisoxazole, thiazole, benzothiazole, isothiazole, imidazole, benzimidazole, pyrazole, indazole, and tetrazole, which are all preferred, as well as furazan, 1,2,3-oxadiazole, 1,2,3-thiadiazole, 1,2,4-thiadiazole,

triazole, benzotriazole, pyridine, quionoline, isoquinoline, pyridazine, pyrimidine, purine, pyrazine, pteridine, and triazine. The most preferred substituents are halo, hydroxy, cyano, lower alkoxy, lower alkyl, lower hydroxyalkyl, lower alkylamino, and lower aminoalkyl.

5 The present invention provides compounds preferably showing a realtively high selectivity toward serotonin receptors, particularly, 5-HT_{2A} receptors, which may have a beneficial effect in the treatment of neuropsychiatric disorders.

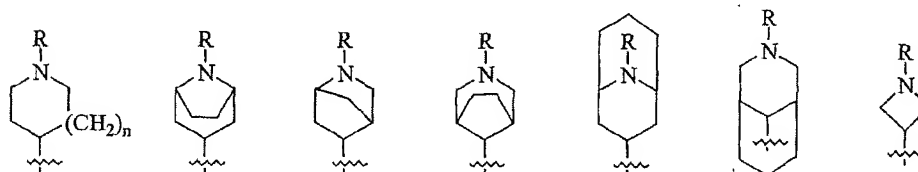
According to one embodiment, the present invention provides compounds of the general formula (I):



I

10 wherein

Z is



or

15 in which

R is a hydrogen, a cyclic or straight-chained or branched acyclic organyl group, a lower hydroxyalkyl group, a lower aminoalkyl group, or an aralkyl or heteroaralkyl group;

n is 0, 1, or 2;

20 X₁ is methylene, vinylene, or an NH or N (lower alkyl) group; and

X₂ is methylene, or, when X₁ is methylene or vinylene, X₂ is methylene or a bond; or when X₁ is methylene, X₂ is O, S, NH, or N(lower alkyl) or a bond;

X

Y₁ is methylene and Y₂ is methylene, vinylene, ethylene, propylene, or a bond;

25 or

Y₁ is a bond and Y₂ is vinylene; or

Y₁ is ethylene and Y₂ is O, S, NH, or N(lower alkyl);

Ar₁ and Ar₂ independently are unsubstituted or substituted aryl or heteroaryl groups; and

W is oxygen or sulfur;

or a pharmacologically acceptable salt, ester, or prodrug thereof.

5 In general, compounds of formula (I) are active at monoamine receptors, specifically serotonin receptors. Preferred compounds share the common property of acting as inverse agonists at the 5-HT_{2A} receptor. Thus, experiments performed on cells transiently expressing the human phenotype of said receptor have shown that the compounds of general formula (I) attenuate the signalling of such receptors in the
10 absence of additional ligands acting upon the receptor. The compounds have thus been found to possess intrinsic activity at this receptor and are able to attenuate the basal, non-agonist-stimulated, constitutive signalling responses that the 5-HT_{2A} receptor displays. The observation that the compounds of general formula (I) are inverse agonists also indicates that these compounds have the ability to antagonize the
15 activation of 5-HT_{2A} receptors that is mediated by endogenous agonists or exogenous synthetic agonist ligands.

In a preferred embodiment, the present invention provides compounds that preferably show a relatively high degree of selectivity towards the 5-HT_{2A} subtype of serotonin receptors relative to other subtypes of the serotonin (5-HT) family of
20 receptors as well as to other receptors, most particularly the monoaminergic G-protein coupled receptors, such as dopamine receptors. In another preferred embodiment, the compounds of the present invention act as inverse agonists at the 5-HT_{2A} subtype of serotonin receptors.

The compounds of general formula (I) may therefore be useful for treating or
25 alleviating symptoms of disease conditions associated with impaired function, in particular elevated levels of activity, of especially 5-HT_{2A} receptors, whether this impaired function is associated with improper levels of receptor stimulation or phenotypical aberrations.

Others have previously hypothesised that certain neuropsychological diseases
30 might be caused by altered levels of constitutive activity of monoamine receptors. Such constitutive activity might be modified via contacting the relevant receptor with a synthetic inverse agonist. By directly testing a large number of centrally acting medicinal compounds with known clinical activity in neuropsychiatric disease, we determined that compounds with antipsychotic efficacy all shared a common

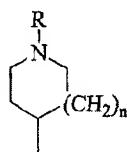
molecular property. Nearly all of these compounds that are used by psychiatrists to treat psychosis were found to be potent 5-HT_{2A} inverse agonists. This correlation is compelling evidence that 5-HT_{2A} receptor inverse agonism is a molecular mechanism of antipsychotic efficacy in humans.

5 Detailed pharmacological characterization of a large number of antipsychotic compounds in our laboratory revealed that they possess broad activity at multiple related receptor subtypes. Most of these compounds display either agonist, competitive antagonist, or inverse agonist activity at multiple monoaminergic receptor subtypes including serotonergic, dopaminergic, adrenergic, muscarinic and
10 histaminergic receptors. This broad activity is likely responsible for the sedating, hypotensive, and motor side effects of these compounds. It follows that the compounds disclosed herein will possess efficacy as, for example, novel antipsychotics, but will have fewer or less severe side effects than existing compounds.

15 The present invention also provides pharmaceutical compositions comprising an effective amount of a compound of general formula (I).

In a preferred embodiment of the compounds of formula (I), Y₁ is methylene and Y₂ is a bond, methylene, ethylene, or vinylene, or Y₁ is ethylene and Y₂ is O or S, and X₁ is methylene and X₂ is a bond, methylene, O, or S, or X₁ is NH or N(lower
20 alkyl).

In a further preferred embodiment of the compounds of formula (I), Z is

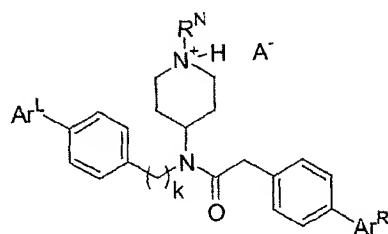


and W is oxygen.

In a more preferred embodiment of the compounds of formula (I), n is 1, Y₁ is methylene, Y₂ is a bond, methylene, ethylene, or vinylene, X₁ is methylene and X₂ is a
25 bond, or X₁ is NH or N(lower alkyl) and X₂ is methylene. In a further preferred embodiment of the compounds of formula (I), W is oxygen and Ar₁ and Ar₂ are different aryl or heteroaryl groups, with different mono-substituted phenyl groups being particularly preferred. Preferably, Ar₁ and Ar₂ are not simultaneously phenyl.

Also preferred compounds of formula (I) are those where Z is 1-(organyl or
30 aralkyl)-4-piperidinyl.

In another embodiment, the invention provides preferred compounds of the formula (II):



II

in which R^N is hydrogen, lower alkyl, aralkyl, or heteroaralkyl;

Ar^L is selected from lower alkyl, lower alkoxy and halogen

Ar^R is selected from lower alkyl, lower alkoxy and halogen;

k is 1 or 2

and A⁻ is a suitable anion.

According to the invention, a suitable anion may be any anion capable of forming a pharmaceutically acceptable salt of the compound, as described in further detail below.

The present invention also provides a method of inhibiting an activity of a monoamine receptor. This method comprises contacting a monoamine receptor or a system containing the monoamine receptor, with an effective amount of a compound of formula (I). According to one embodiment, the monoamine receptor is a serotonin receptor. In a preferred embodiment, the compound is selective for the 5-HT_{2A} receptor subclass. In another preferred embodiment, the compound has little or substantially no activity to other types of receptors, including other serotonergic receptors and most particularly, monoaminergic G-protein coupled receptors, such as dopaminergic receptors.

The system containing the monoamine receptor may, for example, be a subject such as a mammal, non-human primate or a human. The receptor may be located in the central or peripheral nervous system, blood cells or platelets.

The system may also be an in vivo or in vitro experimental model, such as a cell culture model system that expresses a monoamine receptor, a cell-free extract thereof that contains a monoamine receptor, or a purified receptor. Non-limiting examples of such systems are tissue culture cells expressing the receptor or extracts or lysates thereof. Cells that may be used in the present method include any cells

capable of mediating signal transduction via monoamine receptors, especially the 5-HT_{2A} receptor, either via endogenous expression of this receptor (e.g., certain types of neuronal cells lines, for example, natively express the 5-HT_{2A} receptor), or following transfection of cells with plasmids containing the receptor gene. Such cells
5 are typically mammalian cells (or other eukaryotic cells, such as insect cells or *Xenopus* oocytes), because cells of lower organisms generally lack the appropriate signal transduction pathways for the present purpose. Examples of suitable cells include: the mouse fibroblast cell line NIH 3T3 (ATCC CRL 1658), which responds to transfected 5-HT_{2A} receptors by stimulating growth; RAT 1 cells (Pace et al.,
10 *Proc. Natl. Acad. Sci. USA* 88:7031-35 (1991)); and pituitary cells (Vallar et al., *Nature* 330:556-58 (1987)). Other useful mammalian cells for the present method include HEK 293 cells, CHO cells and COS cells.

The invention specifically provides methods of inhibiting an activity of a native, mutated or modified monoamine receptor. Also provided are kits for
15 performing the same. In a preferred embodiment, the activity of the receptor is a signalling activity. In another preferred embodiment, the activity of the receptor is the constitutive basal activity of the receptor. Preferably, the compound is an inverse agonist selective for the 5-HT_{2A} receptor. Most preferably, the compound has little or substantially no activity toward other serotonergic or other monoaminergic
20 receptors, such as dopaminergic receptors.

In one embodiment, the activity of the receptor is a response, such as a signalling response, to an endogenous agonist, such as 5-HT, or an exogenous agonistic agent, such as a drug or other synthetic ligand. The compound of formula (I) preferably acts by inversely agonising or antagonising the receptor.

25 Furthermore, the present invention relates to a method of inhibiting an activation of a monoamine receptor comprising contacting the monoamine receptor, or a system containing the monoamine receptor, with one or more compounds of the invention. The activation of the receptor may be due to an exogenous or endogenous agonist agent, or may be the constitutive activation associated with a native, mutated
30 or modified receptor. The receptor may purified or present in an in vitro or in vivo system. The receptor may also be present in the central or peripheral nervous system, blood cells or platelets of a nonhuman or human subject. Also provided are kits for performing the same.

In a preferred embodiment, the compound is selective for 5-HT class serotonin receptors, more preferably, the 5-HT_{2A} subclass of serotonin receptors. In another preferred embodiment, the compound has little or substantially no anti-dopaminergic activity.

5 The present invention provides methods of treating a disease condition associated with a monoamine receptor comprising administering to a mammal in need of such treatment an effective amount of a compound of formula (I). The invention specifically provides methods for treating or alleviating disease conditions associated with improper function or stimulation of native, as well as mutated or otherwise
10 modified, forms of central serotonin receptors, particularly the 5-HT class of such receptors, comprising administration of an effective amount of a selective inverse agonist of the general formula (I) to a host in need of such treatment. Also provided are kits for performing the same.

 In a preferred embodiment, the receptor is the 5-HT_{2A} subclass. In one
15 embodiment, the disease condition is associated with dysfunction of the serotonin receptor. In another embodiment, the disease condition is associated with activation of the serotonin receptor, preferably inappropriately elevated or constitutive activation, elevated serotonergic tone, as well as disease conditions associated with secondary cellular functions impaired by such pathologies.

20 Examples of diseases for which such treatment using the compounds of the invention, or pharmaceutical compositions comprising such compounds, is useful include, but are not limited to, neuropsychiatric diseases such schizophrenia and related idiopathic psychoses, anxiety, sleep disorders, appetite disorders, affective disorders such as major depression, bipolar disorder, and depression with psychotic
25 features, and Tourette's Syndrome, drug-induced psychoses, psychoses secondary to neurodegenerative disorders such as Alzheimer's or Huntington's Disease. It is anticipated that the compounds of this invention, particularly selective inverse agonists of 5-HT_{2A} that show little or no activity on dopaminergic receptors, may be especially useful for treating schizophrenia. Treatment using the compounds of the
30 invention may also be useful in treating migraine, vasospasm, hypertension, various thrombotic conditions including myocardial infarction, thrombotic or ischemic stroke, idiopathic and thrombotic thrombocytopenic purpura, and peripheral vascular disease.

 In a further embodiment the present invention provides methods for treating or alleviating a disease condition associated with improper function, dysfunction, or

stimulation of native, as well as mutated or otherwise modified, forms of central or peripheral monoamine receptors, such methods comprising administration of an effective amount of a compound of the general formula (I) to a host in need of such treatment. Preferably the monoamine receptor is serotonin receptor in the peripheral
5 nervous system, blood or platelets; more preferably a 5-HT_{2A} subclass receptor. In additional embodiments, the disease condition is associated with increased activity or activation of a serotonin receptor. Also provided are kits for performing the same.

The present invention also pertains to the field of predictive medicine in which pharmacogenomics is used for prognostic (predictive) purposes. Pharmacogenomics
10 deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See e.g., Eichelbaum, *Clin Exp Pharmacol. Physiol.*, 23:983-985 (1996), and Linder, *Clin. Chem.* 43:254-66 (1997). In general, two types of pharmacogenetic conditions can be differentiated: genetic conditions transmitted as a single factor altering the way drugs
15 act on the body (altered drug action), and genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur as naturally occurring polymorphisms.

One pharmacogenomics approach to identifying genes that predict drug response, known as "a genome-wide association," relies primarily on a high-
20 resolution map of the human genome consisting of already known gene-related markers (e.g., a "bi-allelic" gene marker map that consists of 60,000-100,000 polymorphic or variable sites on the human genome, each of which has two variants). Such a high-resolution genetic map can be compared to a map of the genome of each of a statistically significant number of patients taking part in a Phase II/III drug trial to
25 identify markers associated with a particular observed drug response or side effect. Alternatively, such a high-resolution map can be generated from a combination of some ten-million known single nucleotide polymorphisms (SNPs) in the human genome. As used herein, a "SNP" is a common alteration that occurs in a single nucleotide base in a stretch of DNA. For example, a SNP may occur once per every
30 1,000 bases of DNA. A SNP may be involved in a disease process; however, the vast majority may not be disease-associated. Given a genetic map based on the occurrence of such SNPs, individuals can be grouped into genetic categories depending on a particular pattern of SNPs in their individual genome. In such a manner, treatment

regimens can be tailored to groups of genetically similar individuals, taking into account traits that may be common among such genetically similar individuals.

Alternatively, a method termed the "candidate gene approach" can be utilized to identify genes that predict drug response. According to this method, if a gene that
5 encodes a drug's target is known (e.g., a protein or a receptor of the present invention), all common variants of that gene can be fairly easily identified in the population and it can be determined if having one version of the gene versus another is associated with a particular drug response.

Alternatively, a method termed the "gene expression profiling", can be utilized
10 to identify genes that predict drug response. For example, the gene expression of an animal dosed with a drug (e.g., a molecule or modulator of the present invention) can give an indication whether gene pathways related to toxicity have been turned on.

Information generated from more than one of the above pharmacogenomics approaches can be used to determine appropriate dosage and treatment regimens for
15 prophylactic or therapeutic treatment of an individual. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with a molecule or modulator of the invention, such as a modulator identified by one of the exemplary screening assays described herein. As we have described previously, this
20 approach can also be used to identify novel candidate receptor or other genes suitable for further pharmacological characterization in vitro and in vivo.

Accordingly, the present invention also provides methods and kits for identifying a genetic polymorphism predisposing a subject to being responsive to a compound described herein. The method comprises administering to a subject an
25 effective amount of a compound; identifying a responsive subject having an ameliorated disease condition associated with a monamine receptor; and identifying a genetic polymorphism in the responsive subject, wherein the genetic polymorphism predisposes a subject to being responsive to the compound. It is anticipated that this method may be useful both for predicting which individuals are responsive to
30 therapeutic effects of a compound and also for predicting those likely to experience adverse side effect responses. This approach may be useful for identifying, for example, polymorphisms in a serotonin receptor that lead to constitutive activation and are thus amenable to inverse agonist therapy. In addition, this method may be useful for identifying polymorphisms that lead to altered drug metabolism whereby

toxic byproducts are generated in the body. Such a mechanism has been implicated in the rare, but potentially life threatening side effects of the atypical antipsychotic, clozapine.

In a related embodiment, a method for identifying a subject suitable for treatment with a compound of the present invention is provided. According to the method, the presence of a polymorphism that predisposes the subject to being responsive to the compound is detected, the presence of the polymorphism indicating that the subject is suitable for treatment. Also provided are kits for performing the same.

The compounds of this invention preferably show selective inverse agonist activity towards the 5-HT_{2A} receptor. Such activity is defined by an ability of the ligand to attenuate or abolish the constitutive signaling activity of this receptor. Selectivity in the present context is understood as a property of a compound of the invention whereby an amount of compound that effectively inversely agonizes the 5-HT_{2A} receptor and thereby decreases its activity causes little or no inverse agonistic or antagonistic activity at other, related or unrelated, receptors. In particular, the compounds of the invention have surprisingly been found not to interact strongly with other serotonin receptors (5-HT 1A, 1B, 1D, 1E, 1F, 2B, 2C, 4A, 6, and 7) at concentrations where the signaling of the 5-HT_{2A} receptor is strongly or completely inhibited. Preferably, the compounds of the invention are also selective with respect to other monoamine-binding receptors, such as the dopaminergic, histaminergic, adrenergic and muscarinic receptors.

A particularly preferred embodiment of this invention includes:

N-(1-(1-methylethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;

N-(1-(2,2-dimethylethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;

N-(1-pentylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;

N-(1-hexylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;

N-(1-cyclohexylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;

- N-(1-cyclopentylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-cyclobutylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- 5 N-(1-cyclopropylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-(cyclopentylmethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-(cyclobutylmethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- 10 N-(1-(cyclopropylmethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-(2-hydroxyethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- 15 N-(1-(3-hydroxypropyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-((4-Methylphenyl)methyl)-N-(piperidin-4-yl)-N'-phenylmethylcarbamide;
- N-((4-Methylphenyl)methyl)-N-(1-(2-methylpropyl)piperidin-4-yl)-N'-phenylmethylcarbamide;
- 20 N-(1-((2-Bromophenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide;
- N-(1-((4-Hydroxy-3-methoxyphenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide;
- N-(1-((5-Ethylthien-2-yl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide;
- 25 N-(1-(Imidazol-2-ylmethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide;
- N-(1-(Cyclohexylmethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide;
- 30 N-(1-((4-Fluorophenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide;
- N-((4-Methylphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
- N-((4-Methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-4-methoxyphenylacetamide;

- N-(1-Ethylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-((4-Methylphenyl)methyl)-N-(1-propylpiperidin-4-yl)-4-methoxyphenylacetamide;
- 5 N-(1-Butylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-(3,3-Dimethylbutyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- 10 N-(1-(Cyclohexylmethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-((4-Methylphenyl)methyl)-N-(1-(2-methylpropyl)piperidin-4-yl)-4-methoxyphenylacetamide;
- N-((4-Methylphenyl)methyl)-N-(1-((4-methylphenyl)methyl)piperidin-4-yl)-4-methoxyphenylacetamide;
- 15 N-(1-((4-Hydroxyphenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-((2-Hydroxyphenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(3-Phenylpropyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
- 20 N-(2-Phenylethyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
- N-((2-Methoxyphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
- N-((2-Chlorophenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
- N-((3,4-Di-methoxyphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
- 25 N-((4-Fluorophenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
- N-((2,4-Di-chlorophenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
- N-((3-Methylphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
- 30 N-((3-Bromophenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
- N-(1-(Phenylmethyl)piperidin-4-yl)-N-(3-phenyl-2-propen-1-yl)-4-methoxyphenylacetamide;
- N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-phenylacetamide;
- N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-3-phenylpropionamide;

- N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-(phenylthio)acetamide;
N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-phenoxyacetamide;
N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-(4-chlorophenoxy)acetamide;
- 5 N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-3-methoxyphenylacetamide;
N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-4-fluorophenylacetamide;
N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-2,5-dimethoxyphenylacetamide;
- 10 N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-4-chlorophenylacetamide;
N-((4-Methylphenyl)methyl)-N-(1-(phenylmethyl)pyrrolidin-3-yl)-N'-phenylmethylcarbamide;
N-((4-Methylphenyl)methyl)-N-(1-(phenylmethyl)pyrrolidin-3-yl)-4-methoxyphenylacetamide;
- 15 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(piperidin-4-yl) acetamide;
2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide;
2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(1-ethylpiperidin-4-yl) acetamide;
- 20 2-(4-methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-ethylpiperidin-4-yl) acetamide;
2-(4-methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-isopropylpiperidin-4-yl) acetamide;
2-(4-methoxyphenyl)-N-(4-chlorobenzyl)-N-(piperidin-4-yl) acetamide;
2-(4-methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-cyclopentylpiperidin-4-yl) acetamide;
- 25 2-(4-methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-isopropylpiperidin-4-yl) acetamide;
2-(phenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide;
- 30 2-(4-fluorophenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide;
2-(4-Methoxyphenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide;

- 2-(4-Trifluoromethylphenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide;
- 2-(4-Fluorophenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide;
- 2-(4-Methoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl)
- 5 acetamide;
- 2-(phenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide;
- 2-(4-Trifluoromethylphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide;
- 2-(4-trifluoromethylphenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-
- 10 methylpiperidin-4-yl) acetamide;
- 2-Phenyl-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide;
- 2-(4-Chlorophenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide;
- 15 2-(4-Methoxyphenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide;
- 2-(4-trifluoromethylphenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide;
- 2-Phenyl-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl)
- 20 acetamide;
- 2-(4-Chlorophenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide;
- 2-(4-Methoxyphenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide;
- 25 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(4-chloromethyl-2-thiazolylmethyl) piperidin-4-yl] acetamide;
- 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[3(1,3 dihydro-2H-benzimidazol-2-on-1-yl) propyl] piperidin-4-yl} acetamide;
- 2-(4-methoxyphenyl)-N-(2-(4-fluorophenyl) ethyl)-N-(1-methylpiperidin-4-yl)
- 30 acetamide;
- 2-(4-methoxyphenyl)-N-[2-(2,5-dimethoxyphenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide;
- 2-(4-methoxyphenyl)-N-[2-(2,4-dichlorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide;

- 2-(4-methoxyphenyl)-N-[2-(3-chlorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide;
- 2-(4-methoxyphenyl)-N-[2-(4-methoxyphenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide;
- 5 2-(4-methoxyphenyl)-N-[2-(3-fluorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide;
- 2-(4-ethoxyphenyl)-N-[2-(4-fluorophenethyl)-N-(1-methylpiperidin-4-yl) acetamide;
- 2-(4-ethoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide;
- 10 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[2-(2-hydroxyethoxy)ethyl] piperidin-4-yl} acetamide;
- 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-((2-chloro-5-thienyl)methyl) piperidin-4-yl] acetamide;
- 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(2-(imidazolidinon-1-yl)ethyl)piperidin-4-yl] acetamide;
- 15 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[2-(2,4(1H,3H)quinazolinedion-3-yl)ethyl] piperidin-4-yl} acetamide;
- 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[2-(1,3-dioxolan-2-yl)ethyl]piperidin-4-yl} acetamide;
- 20 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[2-(3-indolyl)ethyl] piperidin-4-yl} acetamide;
- 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[3-(1,2,4-triazol-1-yl)propyl]piperidin-4-yl} acetamide;
- 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(5-benzofurazanylmethyl)piperidin-4-yl] acetamide;
- 25 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(5-chlorobenzo[b]thien-3-ylmethyl) piperidin-4-yl] acetamide;
- 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(5-phenyl-1,2,4-oxadiazol-3-ylmethyl)piperidin-4-yl] acetamide;
- 30 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-isopropylpiperidin-4-yl)-acetamide;
- 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-ethylpiperidin-4-yl)-acetamide;
- 2-Phenyl-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide, 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide;

- 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-cyclopentylpiperidin-4-yl)-
acetamide;
- 2-(4-Fluorophenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-
acetamide;
- 5 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-(2-hydroxyethyl)-piperidin-4-
yl)-acetamide;
- 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-cyclobutylpiperidin-4-yl)-
acetamide;
- 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-cyclobutylpiperidin-4-yl)-
10 acetamide;
- 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(tropin-4-yl)-acetamide;
N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-benzyl-carbamide;
N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-phenyl-carbamide;
N-Phenethyl-N-(1-methylpiperidin-4-yl)-N'-benzyl-carbamide;
- 15 2-Phenyl-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-acetamide;
2-(4-Trifluoromethylphenyl)-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-
yl)-acetamide;
- 2-(4-Fluorophenyl)-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-
acetamide;
- 20 2-(4-Methoxyphenyl)-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-
acetamide;
- 2-(4-Methylphenyl)-N-(4-chlorobenzyl)-N-(1-methylpiperidin-4-yl)-
acetamide;
- 2-(4-Hydroxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-
25 acetamide;
- N-Phenethyl-N-(1-methylpiperidin-4-yl)-N'-phenyl-carbamide;
N-(3-Phenylpropyl)-N-(1-methylpiperidin-4-yl)-N'-benzyl-carbamide;
N-(3-Phenylpropyl)-N-(1-methylpiperidin-4-yl)-N'-phenyl-carbamide;
2-(4-Methoxyphenyl)-2,2-ethylene-N-(4-methylbenzyl)-N-(1-methylpiperidin-
30 4-yl) acetamide;
- 2-(4-Methoxyphenyl)-N-alpha-methylbenzyl-N-(1-methylpiperidin-4-yl)
acetamide;
- 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(3-tropen-4-yl) acetamide;
2-Phenyl-2-ethyl-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide;

- N-Phenethyl-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-amine;
 2-(4-Methoxyphenyl)-N-(1-indanyl)-N-(1-methylpiperidin-4-yl) acetamide;
 N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-(4-methoxybenzyl)-
 carbamide;
- 5 2-(3,4-dimethoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)
 acetamide;
 2-(3,4-Methylenedioxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-
 yl) acetamide;
 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-t-butylpiperidin-4-yl)-
 10 acetamide;
 N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-phenethyl-carbamide;
 N-Phenethyl-N-(1-methylpiperidin-4-yl)-N'-phenethyl-carbamide;
 N-(4-Methylbenzyl)-N-(1-t-butylpiperidin-4-yl)-N'-(4-methoxybenzyl)-
 carbamide;
- 15 2-(4-Ethoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)
 acetamide;
 2-(4-Butoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)
 acetamide;
 2-(4-i-Propoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)
 20 acetamide;
 2-(4-t-Butoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)
 acetamide;
 2-(4-Butoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl)
 acetamide;
 25 2-(4-Propoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl)
 acetamide;
 2-(4-i-Propoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl)
 acetamide;
 and, 2-(4-t-Butoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl)
 30 acetamide.

Suitable pharmaceutically acceptable salts of the compounds of this invention include acid addition salts that may, for example, be formed by mixing a solution of the compound according to the invention with a solution of a pharmaceutically acceptable acid such as hydrochloric acid, sulphuric acid, fumaric acid, maleic acid,

succinic acid, acetic acid, benzoic acid, oxalic acid, citric acid, tartaric acid, carbonic acid or phosphoric acid. Furthermore, where the compounds of the invention carry an acidic moiety, suitable pharmaceutically acceptable salts thereof may include alkali metal salts, e.g., sodium or potassium salts; alkaline earth metal salts, e.g., calcium or magnesium salts; and salts formed with suitable organic ligands, e.g., quaternary ammonium salts. Examples of pharmaceutically acceptable salts include the acetate, benzenesulfonate, benzoate, bicarbonate, bisulfate, bitartrate, borate, bromide, calcium, carbonate, chloride, clavulanate, citrate, dihydrochloride, fumarate, gluconate, glutamate, hydrobromide, hydrochloride, hydroxynaphthoate, iodide, isothionate, lactate, lactobionate, laurate, maleate, mandelate, mesylate, methylbromide, methylnitrate, methylsulfate, nitrate, N-methylglucamine ammonium salt, oleate, oxalate, phosphate/diphosphate, salicylate, stearate, sulfate, succinate, tannate, tartrate, tosylate, triethiodide and valerate salt.

The present invention includes within its scope prodrugs of the compounds of this invention. In general, such prodrugs are inactive derivatives of the compounds of this invention that are readily convertible in vivo into the required compound. Conventional procedures for the selection and preparation of suitable prodrug derivatives are described, for example, in *Design of Prodrugs*, (ed. H. Bundgaard, Elsevier, 1985). Metabolites of these compounds include active species that are produced upon introduction of compounds of this invention into the biological milieu.

Where the compounds according to the invention have at least one chiral center, they may exist as a racemate or as enantiomers. It should be noted that all such isomers and mixtures thereof are included in the scope of the present invention. Furthermore, some of the crystalline forms for compounds of the present invention may exist as polymorphs and as such are intended to be included in the present invention. In addition, some of the compounds of the present invention may form solvates with water (i.e., hydrates) or common organic solvents. Such solvates are also included in the scope of this invention.

Where the processes for the preparation of the compounds according to the invention give rise to mixtures of stereoisomers, such isomers may be separated by conventional techniques such as preparative chiral chromatography. The compounds may be prepared in racemic form or individual enantiomers may be prepared by stereoselective synthesis or by resolution. The compounds may be resolved into their component enantiomers by standard techniques, such as the formation of

diastereomeric pairs by salt formation with an optically active acid, such as (-)-di-p-toluoyl-d-tartaric acid and/or (+)-di-p-toluoyl-l-tartaric acid, followed by fractional crystallization and regeneration of the free base. The compounds may also be resolved by formation of diastereomeric esters or amides followed by
5 chromatographic separation and removal of the chiral auxiliary.

Compounds of the present invention may be administered in any of the foregoing compositions and according to dosage regimens established in the art whenever specific pharmacological modification of the activity of monoamine receptors is required.

10 The present invention also provides pharmaceutical compositions comprising one or more compounds of the invention together with a pharmaceutically acceptable diluent or excipient. Preferably such compositions are in unit dosage forms such as tablets, pills, capsules (including sustained-release or delayed-release formulations), powders, granules, elixirs, tinctures, syrups and emulsions, sterile parenteral solutions
15 or suspensions, aerosol or liquid sprays, drops, ampoules, auto-injector devices or suppositories; for oral, parenteral (e.g., intravenous, intramuscular or subcutaneous), intranasal, sublingual or rectal administration, or for administration by inhalation or insufflation, and may be formulated in an appropriate manner and in accordance with accepted practices such as those disclosed in *Remington's Pharmaceutical Sciences*,
20 (Gennaro, ed., Mack Publishing Co., Easton PA, 1990, herein incorporated by reference). Alternatively, the compositions may be in sustained-release form suitable for once-weekly or once-monthly administration; for example, an insoluble salt of the active compound, such as the decanoate salt, may be adapted to provide a depot preparation for intramuscular injection. The present invention also contemplates
25 providing suitable topical formulations for administration to, e.g., eye or skin or mucosa.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic, pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Moreover, when
30 desired or necessary, suitable binders, lubricants, disintegrating agents, flavoring agents and coloring agents can also be incorporated into the mixture. Suitable binders include, without limitation, starch, gelatin, natural sugars such as glucose or beta-lactose, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like. Lubricants used in

these dosage forms include, without limitation, sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum and the like.

5 For preparing solid compositions such as tablets, the active ingredient is mixed with a suitable pharmaceutical excipient such as the ones described above and other pharmaceutical diluents, e.g., water, to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention or a pharmaceutically acceptable salt thereof. By the term "homogeneous" is meant that
10 the active ingredient is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules. The solid preformulation composition may then be subdivided into unit dosage forms of the type described above containing from 0.1 to about 50 mg of the active ingredient of the present invention. The tablets or pills of
15 the present composition may be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner core containing the active compound and an outer layer as a coating surrounding the core. The outer coating may be an enteric layer that serves to resist disintegration in the stomach and permits the inner core to pass intact into the
20 duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings including a number of polymeric acids and mixtures of polymeric acids with conventional materials such as shellac, cetyl alcohol and cellulose acetate.

 The liquid forms in which the present compositions may be incorporated for
25 administration orally or by injection include aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, and flavored emulsions with edible oils such as cottonseed oil, sesame oil, coconut oil or peanut oil, as well as elixirs and similar pharmaceutical carriers. Suitable dispersing or suspending agents for aqueous suspensions include synthetic and natural gums such as tragacanth, acacia, alginate,
30 dextran, sodium carboxymethylcellulose, gelatin, methylcellulose or polyvinylpyrrolidone. Other dispersing agents that may be employed include glycerin and the like. For parenteral administration, sterile suspensions and solutions are desired. Isotonic preparations that generally contain suitable preservatives are employed when intravenous administration is desired. The compositions can also be

formulated as an ophthalmic solution or suspension formation, i.e., eye drops, for ocular administration

Consequently, the present invention also relates to a method of alleviating or treating a disease condition in which modification of monoamine receptor activity, in particular 5-HT_{2A} serotonergic receptor activity, has a beneficial effect by
5 administering a therapeutically effective amount of a compound of the present invention to a subject in need of such treatment. Such diseases or conditions may, for instance arise from inappropriate stimulation or activation of serotonergic receptors. It is anticipated that by using compounds that are selective for a particular serotonin
10 receptor subtype, in particular 5-HT_{2A}, the problems with adverse side effects observed with the known antipsychotic drugs, such as extrapyramidal effects, may be avoided substantially.

The term "therapeutically effective amount" as used herein means an amount of an active compound or pharmaceutical agent that elicits the biological or medicinal
15 response in a tissue, system, animal or human that is being sought by a researcher, veterinarian, medical doctor or other clinician, which includes alleviation of the symptoms of the disease being treated.

Advantageously, compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses, for
20 example, two, three or four times daily. Furthermore, compounds of the present invention may be administered in intranasal form via topical use of suitable intranasal vehicles, via transdermal routes, using those forms of transdermal skin patches well known to persons skilled in the art, by implantable pumps; or by any other suitable means of administration. To be administered in the form of a transdermal delivery
25 system, for example, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated;
30 the route of administration; the renal and hepatic function of the patient; and the particular compound employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the disease or disorder that is being treated.

The daily dosage of the products may be varied over a wide range from about 0.01 mg to about 100 mg per adult human per day. For oral administration, the compositions are preferably provided in the form of tablets containing 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0 or 50.0 mg of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. A unit dose typically contains from about 0.001 mg to about 50 mg of the active ingredient, preferably from about 1 mg to about 10 mg of active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level of from about 0.0001 mg/kg to about 25 mg/kg of body weight per day. Preferably, the range is from about 0.001 to 10 mg/kg of body weight per day, and especially from about 0.001 mg/kg to 1 mg/kg of body weight per day.

Compounds according to the present invention may be used alone at appropriate dosages defined by routine testing in order to obtain optimal pharmacological effect on a monoaminergic receptor, in particular the 5-HT_{2A} serotonergic receptor subtype, while minimizing any potential toxic or otherwise unwanted effects. In addition, co-administration or sequential administration of other agents that improve the effect of the compound may, in some cases, be desirable.

The pharmacological properties and the selectivity of the compounds of this invention for specific serotonergic receptor subtypes may be demonstrated by a number of different assay methods using recombinant receptor subtypes, preferably of the human receptors if these are available, e.g. conventional second messenger or binding assays. A particularly convenient functional assay system is the receptor selection and amplification assay disclosed in U.S. Pat. No. 5,707,798, which describes a method of screening for bioactive compounds by utilizing the ability of cells transfected with receptor DNA, e.g., coding for the different serotonergic subtypes, to amplify in the presence of a ligand of the receptor. Cell amplification is detected as increased levels of a marker also expressed by the cells.

Methods of preparation

The compounds in accordance with the present invention may be synthesized by methods described below, or by modification of these methods. Ways of modifying the methodology include, among others, temperature, solvent, reagents etc, and will be obvious to those skilled in the art.

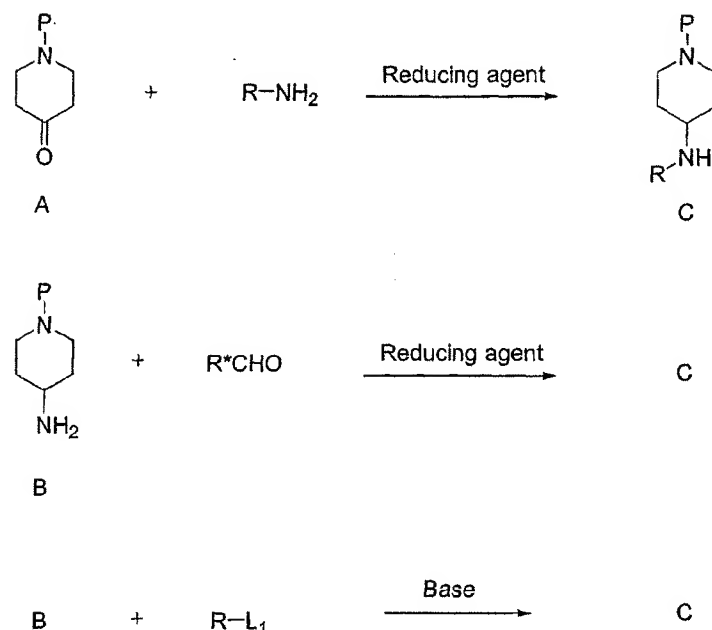
For instance, compounds of the formula C may be synthesized from the corresponding ketone A by reductive amination utilizing any primary amine. The

reaction is conveniently carried out by stirring the reactants in an inert solvent such as methanol or ethanol containing acetic acid. As reducing agent NaBH_4 , NaCNBH_3 , BH_3 ·pyridine or any related reagent may be used including solid-supported reagents. The reaction is typically carried out at room temperature. The ketone A, as
5 exemplified by the piperidone, may be chosen from a list of compounds corresponding to the Z-group listed in formula (I). The ketones can either be obtained commercially or synthesized by methodology disclosed in Lowe et al. *J. Med. Chem.* 37: 2831-40 (1994); Carroll et al. *J. Med. Chem.* 35:2184-91 (1992); or Rubiralta et al. *Piperidine – Structure, Perparation, Reactivity and Synthetic Applications of*
10 *Piperidine and its Derivatives. (Studies in Organic Chemistry 43, Elsevier, Amsterdam, 1991).* The protecting group P includes groups such as those described in T. W. Greene and P. G. M. Wuts, *Protective Groups in Organic Chemistry*, 3. Ed. John Wiley & Sons, 1999, and they should be chosen in such a way, that they are stable to the reaction conditions applied and readily removed at a convenient stage
15 using methodology known from the art. Typical protecting groups are N-Boc, N-Cbz, N-Bn.

Alternatively, the amine C can be synthesized from the primary amine B by reductive amination with any aldehyde. The reaction is conveniently carried out by stirring the reactants in an inert solvent such as methanol or ethanol containing acetic
20 acid. As reducing agent NaBH_4 , NaCNBH_3 , BH_3 ·pyridine or any related reagent may be used including solid-supported reagents. The reaction is typically carried out at room temperature. The primary amine B, as exemplified by the 4-aminopiperidine, may be chosen from a list of compounds corresponding to the Z-groups listed in formula (I). The amines can either be obtained commercially or synthesized from the
25 corresponding ketones. The protecting group P may be chosen as stated above.

Alternatively, the amine C can be synthesized from the primary amine B by alkylation with any alkylating agent (R-L_1). The leaving group L_1 is suitably a halogen atom, e.g., bromine or iodine, or a sulfonate, e.g. tosylate or mesylate, or another leaving group favoring the reaction. The reaction is conveniently carried out
30 by stirring the reagents under basic conditions in an inert solvent, e.g., diisopropylethylamine in acetonitrile, or K_2CO_3 in *N,N*-dimethylformamide. The reaction is typically carried out at temperatures between room temperature and 80°C . The primary amine B, as exemplified by the 4-aminopiperidine, may be chosen from a list of compounds corresponding to the Z-groups listed in formula (I). The amines

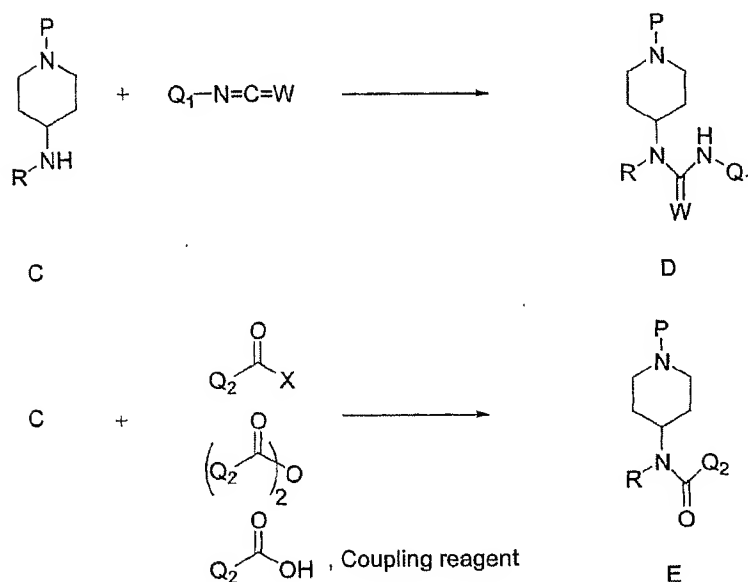
can either be obtained commercially or synthesized from the corresponding ketones. The protecting group P may be chosen as stated above.



5 Wherein R and R* are defined in agreement with formula (I), and P represents a suitable protecting group, and L₁ represents a suitable leaving group.

The secondary amine C may be acylated using any isocyanate or isothiocyanate (Q₁-N=C=W) to give the corresponding ureas or thioureas D. The reaction is typically carried out by stirring the reactants, using an excess of isocyanate or isothiocyanate in an inert solvent, e.g., dichloromethane at a temperature between 0°C and room temperature and under dry conditions. The amine C may also be acylated using any carboxylic acid halide (Q₂COX), e.g., chloride, or carboxylic anhydride ((Q₂C=O)₂O) to give amides of the general structure E. The reaction is typically carried out using an excess of the acylating agent and a suitable base, e.g., triethylamine or diisopropylethylamine in an inert solvent, e.g., dichloromethane, at a temperature between 0°C and room temperature and under dry conditions. As an alternative to the carboxylic acid halides and carboxylic acid anhydrides, the amine C may be acylated using a carboxylic acid (Q₂COOH) and a suitable coupling reagent e.g. DCC or EDCI. The reaction is typically carried out using an excess of the

acylating agent and the coupling reagent in an inert solvent, e.g., dichloromethane at a temperature between 0°C and room temperature and under dry conditions. The compounds of the general structure (E) may be converted into the corresponding thioamides using methodology disclosed in Varma et al., *Org. Lett.* 1: 697-700 (1999); Cherkasov et al. *Tetrahedron* 41:2567 (1985); or Scheibye et al, *Bull. Soc. Chim. Belg.* 87:229 (1978).



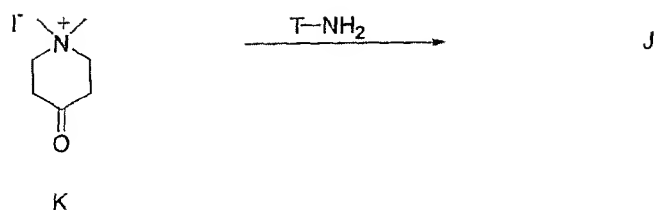
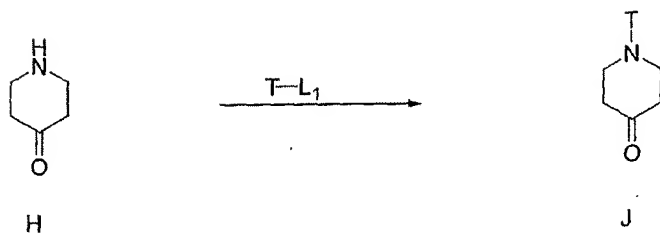
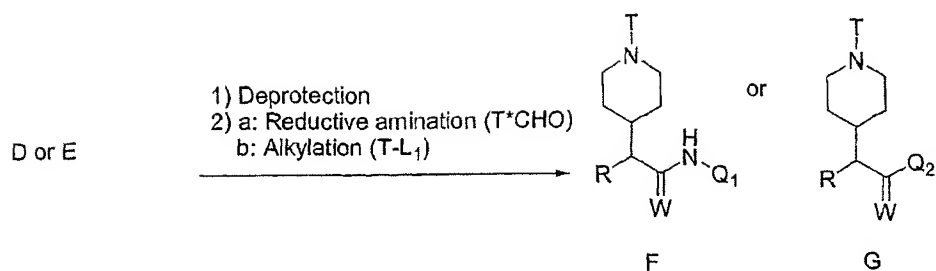
Wherein R, Q₁, Q₂, and W are defined in agreement with formula (I), P represents a suitable protecting group, and X represents a halide.

The substituent G on the ring nitrogen can be introduced by a two step procedure. First, the protecting group on the urea D or the amide E is removed using well-known methods. For example, the N-Boc group is removed by treating the protected compound with 4 M HCl in dioxane or trifluoroacetic acid in dichloromethane. Second, the secondary amines obtained from D and E can be alkylated by reductive amination using any aldehyde (T-CHO). The reaction is conveniently carried out by stirring the reactants in an inert solvent such as methanol or ethanol. As a reducing agent, solid-supported borohydride, NaBH₄, NaCNBH₃, BH₃·pyridine or any related reagent may be used, including solid-supported reagents. The reaction is typically carried out at room temperature.

Alternatively, the compounds F and G can be synthesized from the secondary amine obtained from D or E as described above by alkylation with any alkylating agent (T- L₁). The leaving group L₁ is suitably a halogen atom, e.g., bromine or iodine, or a sulfonate, e.g., tosylate or mesylate, or another leaving group favoring the reaction. The reaction is conveniently carried out by stirring the reagents under basic conditions in an inert solvent, for example diisopropylethylamine in acetonitrile, or K₂CO₃ in *N,N*-dimethylformamide. The reaction is typically carried out at temperatures between room temperature and 80°C.

Alternatively, the T-group can be introduced in the first step of the synthetic sequence leading to the compounds in accordance with the present invention by *N*-alkylation of compound H with any alkylating agent (T- L₁). The leaving group L₁ is suitably a halogen atom, e.g., bromine or iodine, or a sulfonate, e.g., tosylate or mesylate, or another leaving group favoring the reaction. The reaction is conveniently carried out by stirring the reagent under basic conditions in an inert solvent, e.g., diisopropylethylamine in acetonitrile, or K₂CO₃ in *N,N*-dimethylformamide. The reaction is typically carried out at temperatures between room temperature and 80°C. The secondary amine H, as exemplified by 4-piperidone, may be chosen from a list of compounds corresponding to the Z-groups listed in formula (I). The amines can either be obtained commercially or synthesized from methodology disclosed in Lowe et al., *J. Med. Chem.* 37:2831-40 (1994); and Carroll et al., *J. Med. Chem.* 35:2184-91 (1992).

Alternatively, compounds of the general structure J may be synthesized starting from K using the method disclosed in: Kuehne et al., *J. Org. Chem.* 56:2701 (1991); and Kuehne et al., *J. Org. Chem.* (1991), 56:513.



wherein R, Q₁, Q₂, W, and T are defined in agreement with formula (I), and L₁ is a suitable leaving group.

In general, during any of the processes for preparation of the compounds of the present invention, it may be necessary and/or desirable to protect sensitive or reactive groups on any of the molecules concerned. This may be achieved by means of conventional protecting groups, such as those described in *Protective Groups in Organic Chemistry* (ed. J.F.W. McOmie, Plenum Press, 1973); and Greene & Wuts, *Protective Groups in Organic Synthesis*, John Wiley & Sons, 1991. The protecting groups may be removed at a convenient subsequent stage using methods known from the art.

Examples

The invention is disclosed in further detail in the following examples that are not in any way intended to limit the scope of the invention as claimed.

General LC-MS procedure for Examples 1-41: All spectra were obtained using an HP1100 LC/MSD-instrument. A setup with a binary pump, autosampler, column oven, diode array detector, and electrospray ionization interface was used. A reversed phase column (C18 Luna 3mm particle size, 7.5 cm x 4.6 mm ID) with a guard cartridge system was used. The column was maintained at a temperature of 30°C. The mobile phase was acetonitrile/8 mM aqueous ammonium acetate. A 15 minute gradient program was used, starting at 70% acetonitrile, over 12 minutes to 95 % acetonitrile, over 1 minute back to 70% acetonitrile, where it stayed for 2 minutes. The flow rate was 0.6 ml/min. The t_r values reported in the specific examples below were obtained using this procedure.

Example 1 - *N*-((4-Methylphenyl)methyl)-*N*-(piperidin-4-yl)-*N*⁷-phenylmethylcarbamide (26HCH65)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (1.75 g, 8.8 mmol) and 4-methylbenzylamine (970 mg, 8.0 mmol) in methanol (7 ml) was added acetic acid in methanol (1 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 30 ml). The resulting solution was stirred at room temperature. After 20 h, water (5 ml) was added, and the mixture was stirred for 1 h before it was concentrated. Flash chromatography in dichloromethane:methanol 10:1 gave *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate. Yield: 2.4 g, 98%. To a solution of *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate (800 mg, 2.63 mmol) in dry dichloromethane (20 ml) was added benzylisocyanate (0.65 ml, 5.26 mmol). The solution was stirred at room temperature. After 48 h, an excess of 2-dimethylaminoethylamine was added. The mixture was stirred for another 24 h, before it was concentrated. The resulting solid was redissolved in dichloromethane (20 ml), sequentially washed with HCl (0.2 N, 3x30 ml), and water (20 ml), dried (Na₂SO₄), filtered and concentrated. Flash chromatography in dichloromethane:methanol 10:1 gave *N*-((4-methylphenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-*N*⁷-phenylmethylcarbamide (760 mg, 66 %), which was dissolved in diethyl ether (5 ml). HCl (4 M) in dioxane (3 ml) was added, and the solution was stirred at room

temperature for 60 min, then concentrated. The resulting oil was redissolved in a mixture of dichloromethane and diethyl ether (4:1). The organic layer was extracted with HCl (0.2 M, 3×20 ml). The combined aqueous layers were treated with NaOH (0.2 M) until basic (pH>8), then extracted with dichloromethane (3×20 ml). The combined organic layers were dried (Na₂SO₄), filtered, and concentrated to give the title compound. Yield: 406 mg, 70%; ¹³C-NMR (CDCl₃): δ 21.3, 31.6, 45.0, 45.9, 46.4, 53.0, 126.3, 127.2, 127.4, 128.6, 129.8, 135.3, 137.4, 139.7, 158.5.

Example 2 - *N*-((4-Methylphenyl)methyl)-*N*-(1-(2-methylpropyl)piperidin-4-yl)-*N*³-phenylmethylcarbamide (26HCH66-02)

The product from example 1 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). 2-Methylpropionaldehyde (0.08 ml, 0.6 mmol) was added followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. IR: 1640, 1185, 1110 cm⁻¹; LC-MS: (M+H)⁺ 394.2, t_r 5.60 min.

Example 3 - *N*-(1-((2-Bromophenyl)methyl)piperidin-4-yl)-*N*-((4-methylphenyl)methyl)-*N*³-phenylmethylcarbamide (26HCH66-03)

The product from example 1 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). 2-Bromobenzaldehyde (0.07 ml, 0.6 mmol) was added followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. IR: 1635, 1180, 1110 cm⁻¹; LC-MS: (M+H)⁺ 506.1, t_r 8.37 min.

Example 4 - N-(1-((4-Hydroxy-3-methoxyphenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide (26HCH66-04)

The product from example 1 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). 4-Hydroxy-3-methoxybenzaldehyde (91 mg, 0.6 mmol) was added
5 followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g
10 resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. ¹³C-NMR (CD₃OD, selected): δ 19.9, 55.4, 126.5, 127.0, 128.1, 129.0, 140.3, 148.0, 148.1, 158.8.

Example 5 - N-(1-((5-Ethylthien-2-yl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide (26HCH66-05)

The product from example 1 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). 5-Ethyl-2-thiophencarboxaldehyde (84 mg, 0.6 mmol) was added
15 followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g
20 resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. IR: 1640, 1185, 1110, 805, 700, 620 cm⁻¹; LC-MS: (M+H)⁺ 462.3, t_r 7.52 min.

Example 6 - N-(1-(Imidazol-2-ylmethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide (26HCH66-06)

The product from example 1 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). Imidazole-2-carboxaldehyde (58 mg, 0.6 mmol) was added followed
25 by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was

added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. IR: 1620, 1190, 1100, 805, 700, 620 cm⁻¹; LC-MS: (M+H)⁺ 418.2, t_r 2.05 min.

5 **Example 7 - *N*-(1-(Cyclohexylmethyl)piperidin-4-yl)-*N*'-(4-methylphenyl)methyl)-*N*³-phenylmethylcarbamide (26HCH66-09)**

The product from example 1 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). Cyclohexanecarboxaldehyde (67 mg, 0.6 mmol) was added followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. IR: 1635, 1175, 1100, 805, 695, 620 cm⁻¹; LC-MS: (M+H)⁺ 434.4, t_r 7.44 min.

Example 8 - *N*-(1-((4-Fluorophenyl)methyl)piperidin-4-yl)-*N*'-(4-methylphenyl)methyl)-*N*³-phenylmethylcarbamide (26HCH66-10)

The product from example 1 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). 4-Fluorobenzaldehyde (0.08 ml, 0.6 mmol) was added followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. IR: 1640, 1175, 1110, 805, 700, 620 cm⁻¹; LC-MS: (M+H)⁺ 446.3, t_r 5.62 min.

30 **Example 9 - *N*'-(4-Methylphenyl)methyl)-*N*-(1-(phenylmethyl)piperidin-4-yl)-*N*³-phenylmethylcarbamide hydrochloride (26HCH16D)**

To a solution of 1-benzyl-4-piperidone (1.74 g, 9.2 mmol) and 4-methylbenzylamine (0.97 g, 8 mmol) in methanol (30 ml) was added sodium

borohydride (525 mg) in small portions over 30 min. The reaction mixture was stirred at room temperature. After 16 h, the mixture was concentrated. Water (30 ml) was added, and the mixture was extracted with dichloromethane (2×20 ml). The combined organic layers were dried (Na₂SO₄), filtered, and concentrated to give 4-((4-methylphenyl)methyl)amino-1-phenylmethylpiperidine. The crude product was used without further purification.

4-((4-Methylphenyl)methyl)amino-1-phenylmethylpiperidine (800 mg, 2.7 mmol) was dissolved in dry dichloromethane (30 ml). Benzylisocyanate (543 mg, 4.1 mmol) was added. The reaction mixture was stirred at room temperature. After 16 h, water (10 ml) was added followed by NaOH (6 N, 2 ml). After additional 30 minutes of stirring the white crystals were filtered off. The organic layer was isolated and dried (Na₂SO₄), filtered, and concentrated. Flash chromatography in dichloromethane/methanol 10/1 left *N*-((4-Methylphenyl)methyl)-*N*-(1-(phenylmethyl)piperidin-4-yl)-*N'*-phenylmethylcarbamide Yield: 820 mg, 71%; A sample was concentrated with HCl (4 M in dioxane) followed by recrystallization from dichloromethane/diethyl ether leaving the title compound. ¹H-NMR (CDCl₃): δ 1.87 (br d, 2 H), 2.30 (s, 3 H), 2.59 (dq, 2 H), 2.76 (br q, 2 H), 3.44 (br d, 2 H), 4.09 (d, 2 H), 4.30 (d, 2 H), 4.40 (s, 2 H), 4.64–4.76 (m, 2 H), 6.98–7.64 (Aromatic protons, 14 H); ¹³C-NMR (CDCl₃): δ 21.2, 26.7, 45.0, 46.0, 49.7, 52.2, 61.0, 126.2, 127.26, 126.31, 128.2, 128.6, 129.6, 129.9, 130.4, 131.6, 134.4, 137.6, 139.3, 158.5; ¹³C-NMR (CD₃OD, rotamers): δ 19.8, 26.4, 27.8, 40.3, 44.3, 51.6, 51.9, 54.5, 60.5, 110.0, 112.1, 114.0, 114.2, 117.5, 125.9, 126.2, 126.7, 126.8, 128.9, 129.1, 129.2, 129.4, 129.7, 130.1, 131.2, 134.5, 137.4, 159.1, 173.8, 175.0; Mp. 109–112°C; Elemental analysis: Found C, 70.06; H, 7.62; N, 8.60; calcd for monohydrate: C, 69.76; H, 7.53; N, 8.72.

Example 10 - *N*-((4-Methylphenyl)methyl)-*N*-(1-(phenylmethyl)piperidin-4-yl)-*N'*-phenylmethylcarbamide oxalate (34JJ59oxal)

N-((4-Methylphenyl)methyl)-*N*-(1-(phenylmethyl)piperidin-4-yl)-*N'*-phenylmethylcarbamide was prepared as described in example 9 above. A sample was precipitated as the oxalate and recrystallized from ethyl acetate to give the title compound. ¹³C-NMR (CDCl₃): δ 21.2, 27.0, 45.0, 45.9, 49.9, 52.1, 60.6, 126.1, 127.3, 127.4, 128.5, 128.7, 129.6, 130.0, 130.4, 131.2, 134.3, 137.7, 139.3, 158.4,

163.4; Mp. 180-182°C; Elemental analysis: Found C, 69.54; H, 6.73; N, 7.96; calcd for monooxalate: C, 69.61; H, 6.82; N, 8.12.

Example 11 - N-((4-Methylphenyl)methyl)-N-(1-(phenylmethyl)piperidin-4-yl)-4-methoxyphenylacetamide hydrochloride (26HCH17)

5 To a solution of 1-benzyl-4-piperidone (1.74 g, 9.2 mmol) and 4-methylbenzylamine (0.97 g, 8 mmol) in methanol (30 ml) was added sodium borohydride (525 mg) in small portions over 30 min. The reaction mixture was stirred at room temperature. After 16 h, the mixture was concentrated. Water (30 ml) was added, and the mixture was extracted with dichloromethane (2×20 ml). The combined
10 organic layers were dried (Na₂SO₄), filtered, and concentrated to give 4-((4-methylphenyl)methyl)amino-1-phenylmethylpiperidine. The crude product was used without further purification.

To a solution of 4-((4-Methylphenyl)methyl)amino-1-phenylmethylpiperidine (800 mg, 2.7 mmol) in dry dichloromethane (30 ml) was added diisopropylethylamine
15 (1.5 ml) followed by 4-methoxyphenylacetyl chloride (997 mg, 5.4 mmol). The reaction mixture was stirred at room temperature. After 16 h, the reaction mixture was concentrated, redissolved in diethyl ether, and extracted with HCl (0.6 N). The aqueous layer was isolated, treated with NaOH (1 N) until basic, and extracted with dichloromethane (20 ml). The organic layer was isolated and dried (Na₂SO₄),
20 filtered, and concentrated, and redissolved in diethyl ether. The hydrochloride was formed by addition of HCl (4 M in dioxane), and recrystallized from diethyl ether to give the title compound. Yield: 600 mg, 50%; ¹H-NMR (CDCl₃): δ 1.75 (d, 2 H), 2.32 (s, 3 H), 2.50 (q, 2 H), 2.70 (q, 2 H), 3.38 (d, 2 H), 3.54 (s, 2 H), 3.78 (s, 3 H), 4.06 (d, 2 H), 4.54 (s, 2 H), 4.82 (m, 1 H), 6.78-7.60 (aromatic protons, 13 H); ¹³C-
25 NMR (CDCl₃): δ 21.0, 26.0, 40.3, 46.3, 49.0, 51.8, 55.3, 60.8, 114.2, 125.6, 126.6, 127.9, 129.4, 129.60, 129.62, 130.3, 131.4, 134.8, 137.1, 158.7, 172.9; Mp. 197-200°C; Elemental analysis: Found C, 71.29; H, 7.25; N, 5.73; calcd for hydrate: C, 71.37; H, 7.43; N, 5.74.

Example 12 - N-((4-Methylphenyl)methyl)-N-(1-(phenylmethyl)piperidin-4-yl)-4-methoxyphenylacetamide oxalate (34JJ61oxal)

30 N-((4-Methylphenyl)methyl)-N-(1-(phenylmethyl)piperidin-4-yl)-4-methoxyphenylacetamide was prepared as described in example 11 above. A sample

was precipitated as the oxalate and recrystallized from tetrahydrofuran to give the title compound. ^{13}C -NMR (CDCl_3): δ 21.2, 26.4, 40.6, 52.0, 55.5, 114.4, 125.9, 126.7, 128.4, 129.6, 129.8, 129.9, 130.4, 131.2, 134.6, 137.6, 158.9, 163.3, 172.9; Mp. 171-173°C; Elemental analysis: Found C, 69.56; H, 6.74; N, 5.16; calcd for monooxalate:
5 C, 69.48; H, 6.61; N, 5.40.

Example 13 - N-((4-Methylphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide (26HCH71B)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (1.75 g, 8.8 mmol) and 4-methylbenzylamine (970 mg, 8.0 mmol) in
10 methanol (7 ml) was added acetic acid in methanol (1 M, 6.7 ml) followed by NaCNBH_3 in methanol (0.3 M, 30 ml). The resulting solution was stirred at room temperature. After 20 h, water (5 ml) was added, and the mixture was stirred for 1 h before it was concentrated. Flash chromatography in dichloromethane:methanol 10:1 gave *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate. Yield: 2.4 g,
15 98%. To a solution of *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate (862 mg, 2.83 mmol) in dry dichloromethane (10 ml) was added diisopropylethylamine (1.1 ml, 6.5 mmol) followed by 4-methoxyphenylacetyl chloride (0.66 ml, 4.3 mmol). The reaction mixture was stirred at room temperature. After 48 h, water (5 ml) was added, and the mixture was stirred for additional 2 h
20 before extracted with NaOH (0.2 N, 2×15 ml), HCl (0.2 N, 2×15 ml), and water (15 ml). The organic layer was dried (Na_2SO_4) and concentrated to give *N*-((4-methylphenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide. The crude product was used without any further purification. *N*-((4-Methylphenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide was dissolved in ether (2 ml) and HCl (3 ml, 4 M in dioxane) was added. The reaction mixture was stirred at room temperature. After 2 h, water (5 ml) was added, and the mixture was extracted with HCl (0.1 N, 3×30 ml). The combined aqueous layers were treated with NaOH (0.2 N) until basic (pH > 8). The aqueous layer was extracted with diethyl ether (2×20 ml). The combined organic
25 layers were dried (Na_2SO_4) and concentrated, before dissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH_3 in methanol, and concentrated. Additional flash chromatography in

dichloromethane/methanol 1/1→methanol containing 2% NH₃ gave the title compound. Yield: 466mg, 47%; ¹³C-NMR (CD₃OD, rotamers): δ 19.9, 27.8, 29.7, 40.2, 40.3, 44.4, 44.45, 44.50, 52.4, 54.5, 55.5, 114.0, 114.1, 126.0, 126.7, 126.9, 127.3, 128.7, 129.3, 129.6, 129.7, 135.1, 136.1, 136.2, 137.1, 159.0, 159.1, 173.1, 173.7.

Example 14 - *N*-(1-(3,3-Dimethylbutyl)piperidin-4-yl)-*N*-((4-methylphenyl)methyl)-4-methoxyphenylacetamide (26HCH79-5)

The product from example 13 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). 3,3-Dimethylbutyraldehyde (0.143 ml, 1.1 mmol) was added followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 26 mg; ¹³C-NMR (CD₃OD, rotamers): δ 19.9, 27.4, 28.4, 28.8, 29.2, 29.3, 38.3, 38.4, 40.2, 40.3, 44.3, 52.0, 52.3, 52.4, 53.9, 54.6, 54.9, 114.0, 114.1, 126.0, 126.8, 127.0, 127.3, 128.8, 129.4, 129.8, 129.9, 135.0, 136.1, 136.3, 137.1, 158.96, 159.05, 173.2, 173.8.

Example 15 - *N*-(1-(Cyclohexylmethyl)piperidin-4-yl)-*N*-((4-methylphenyl)methyl)-4-methoxyphenylacetamide (26HCH79-6)

The product from example 13 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). Cyclohexanecarboxaldehyde (0.138 ml, 1.1 mmol) was added followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 17 mg; HRMS (FAB⁺, NBA) (M+H)⁺ 449.3163, C₂₉H₄₁N₂O₂ requires 449.3168; LC-MS: (M+H)⁺ 449.2, t_r 7.92 min.

Example 16 - *N*-((4-Methylphenyl)methyl)-*N*-(1-(2-methylpropyl)piperidin-4-yl)-4-methoxyphenylacetamide (26HCH79-7)

The product from example 13 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). 2-Methylpropionaldehyde (0.104 ml, 1.1 mmol) was added followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 19 mg; HRMS (FAB⁺, NBA) (M+H)⁺ 409.2858, C₂₆H₃₇N₂O₂ requires 409.2855; LC-MS: (M+H)⁺ 409.2, t_r 5.97 min.

Example 17 - *N*-((4-Methylphenyl)methyl)-*N*-(1-(4-methylphenyl)methyl)piperidin-4-yl)-4-methoxyphenylacetamide (26HCH79-8)

The product from example 13 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). 4-Methylbenzaldehyde (0.134 ml, 1.1 mmol) was added followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 22 mg; HRMS (FAB⁺, NBA) (M+H)⁺ 457.2853, C₃₀H₃₇N₂O₂ requires 457.2855; LC-MS: (M+H)⁺ 457.2, t_r 6.97 min.

Example 18 - *N*-(1-(4-Hydroxyphenyl)methyl)piperidin-4-yl)-*N*-((4-methylphenyl)methyl)-4-methoxyphenylacetamide (26HCH79-9)

The product from example 13 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). 4-Hydroxybenzaldehyde (139 mg, 1.1 mmol) was added followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h,

the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 19 mg; HRMS (FAB⁺, NBA) (M+H)⁺ 459.2655, C₂₉H₃₅N₂O₃ requires 459.2648; LC-MS: (M+H)⁺ 459.1, t_r 2.84 min.

Example 19 - *N*-(1-((2-Hydroxyphenyl)methyl)piperidin-4-yl)-*N*-((4-methylphenyl)methyl)-4-methoxyphenylacetamide (26HCH79-10)

The product from example 13 above (20 mg, 0.06 mmol) was dissolved in abs. ethanol (2 ml). 2-Hydroxybenzaldehyde (0.122 ml, 1.1 mmol) was added followed by solid-supported borohydride (150 mg, 2.5 mmol/g resin; Aldrich 32,864-2). The mixture was shaken at room temperature. After 48 h, the resin was filtered off and acetic anhydride (0.02 ml, 0.2 mmol) was added to the organic solution. After 24 h, the mixture was concentrated and redissolved in methanol (2 ml). The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 16 mg; HRMS (FAB⁺, NBA) (M+H)⁺ 459.2633, C₂₉H₃₅N₂O₃ requires 459.2648; LC-MS: (M+H)⁺ 459.2, t_r 5.81 min.

Example 20 -*N*-(3-Phenylpropyl)-*N*-(piperidin-4-yl)-4-methoxyphenylacetamide (26HCH80-1)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (400 mg, 2 mmol) in methanol (1 ml) and 3-phenylpropylamine (0.143 ml, 1 mmol) in methanol (1 ml) was added acetic acid in methanol (1 M, 1.34 ml) followed by NaCNBH₃ in methanol (0.3 M, 4.4 ml). The resulting solution was stirred at room temperature. After 24 h, water (2 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. The resulting oil was redissolved in diethyl ether (20 ml), extracted with HCl (0.1 N, 1×15 ml). The aqueous layer was washed with diethyl ether (10 ml) and treated with 0.2 N NaOH until basic (pH>8), before extracted with dichloromethane (20 ml). The organic layer was dried (Na₂SO₄), filtered, and concentrated to give *tert*-butyl 4-(3-phenylpropyl)amino-piperidine carboxylate. Yield: 110 mg. To a solution of *tert*-butyl 4-(3-phenylpropyl)amino-

piperidine carboxylate (50 mg, 0.16 mmol) in dichloromethane (6 ml) was added diisopropylethylamine (0.070 ml, 0.4 mmol) followed by 4-methoxyphenylacetyl chloride (0.055 ml, 0.35 mmol). The reaction mixture was stirred at room temperature. After 18 h, water (2 ml) was added. The mixture was stirred for another
5 2 h. The mixture was sequentially washed with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and water (10 ml), dried (Na₂SO₄), filtered and concentrated to give *N*-(3-phenylpropyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide. The crude product was used without any further purification. *N*-(3-Phenylpropyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide was dissolved in diethyl ether (2 ml) and HCl (1 ml, 4 M in dioxane) was added. The reaction mixture was stirred at room temperature. After 2.5 h, NaOH (1 ml, 6 N) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×15 ml), dried (Na₂SO₄), filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin
15 (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 61 mg; ¹³C-NMR (CD₃OD, rotamers): δ 27.8, 29.4, 30.8, 32.3, 32.7, 33.3, 40.2, 40.5, 42.0, 44.5, 44.6, 44.9, 52.7, 54.56, 54.57, 54.9, 114.0, 114.1, 125.7, 126.1, 127.0, 127.4, 128.2, 128.3, 128.5, 129.47, 129.55, 141.2, 141.8, 158.9, 159.0, 172.5, 172.7.

20 **Example 21 - *N*-(2-Phenylethyl)-*N*-(piperidin-4-yl)-4-methoxyphenylmethylacetamide (26HCH80-2)**

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (400 mg, 2 mmol) in methanol (1 ml) and 2-phenylethylamine (0.143 ml, 1 mmol) in methanol (1 ml) was added acetic acid in methanol (1 M, 1.34 ml)
25 followed by NaCNBH₃ in methanol (0.3 M, 4.4 ml). The resulting solution was stirred at room temperature. After 24 h, water (2 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. The resulting oil was redissolved in diethyl ether (20 ml), extracted with HCl (0.1 N, 1×15 ml). The aqueous layer was washed with diethyl ether (10 ml) and treated with 0.2 N NaOH until basic (pH>8), before
30 extracted with dichloromethane (20 ml). The organic layer was dried (Na₂SO₄), filtered, and concentrated to give *tert*-butyl 4-(2-phenylethyl)amino-piperidine carboxylate. Yield: 221 mg. To a solution of *tert*-butyl 4-(2-phenylethyl)amino-

piperidine carboxylate (50 mg, 0.16 mmol) in dichloromethane (6 ml) was added diisopropylethylamine (0.070 ml, 0.4 mmol) followed by 4-methoxyphenylacetyl chloride (0.055 ml, 0.35 mmol). The reaction mixture was stirred at room temperature. After 18 h, water (2 ml) was added. The mixture was stirred for another
5 2 h. The mixture was sequentially washed with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and water (10 ml), dried (Na₂SO₄), filtered and concentrated to give *N*-(2-phenylethyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide. The crude product was used without any further purification. *N*-(2-Phenylethyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide was dissolved in
10 diethyl ether (2 ml) and HCl (1 ml, 4 M in dioxane) was added. The reaction mixture was stirred at room temperature. After 2.5 h, NaOH (1 ml, 6 N) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×15 ml), dried (Na₂SO₄), filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed
15 with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 52 mg; ¹³C-NMR (CD₃OD, rotamers): δ 27.1, 28.5, 34.9, 36.6, 40.2, 40.4, 44.1, 44.2, 44.4, 53.3, 54.2, 54.6, 114.0, 114.1, 126.2, 126.6, 127.2, 127.4, 128.3, 128.6, 128.79, 128.82, 129.7, 138.5, 139.5, 158.96, 159.0, 172.7, 173.1

20 **Example 22 - *N*-((2-Methoxyphenyl)methyl)-*N*-(piperidin-4-yl)-4-methoxyphenylmethylacetamide (26HCH80-4)**

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (400 mg, 2 mmol) in methanol (1 ml) and 2-methoxybenzylamine (0.130 ml, 1 mmol) in methanol (1 ml) was added acetic acid in methanol (1 M, 1.34 ml)
25 followed by NaCNBH₃ in methanol (0.3 M, 4.4 ml). The resulting solution was stirred at room temperature. After 24 h, water (2 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. The resulting oil was redissolved in diethyl ether (20 ml), extracted with HCl (0.1 N, 1×15 ml). The aqueous layer was washed with diethyl ether (10 ml) and treated with 0.2 N NaOH until basic (pH>8), before
30 extracted with dichloromethane (20 ml). The organic layer was dried (Na₂SO₄), filtered, and concentrated to give *tert*-butyl 4-((2-methoxyphenyl)methyl)amino-piperidine carboxylate. Yield: 211 mg. To a solution of *tert*-butyl 4-((2-

methoxyphenyl)methyl)amino-piperidine carboxylate (50 mg, 0.16 mmol) in dichloromethane (6 ml) was added diisopropylethylamine (0.070 ml, 0.4 mmol) followed by 4-methoxyphenylacetyl chloride (0.055 ml, 0.35 mmol). The reaction mixture was stirred at room temperature. After 18 h, water (2 ml) was added. The mixture was stirred for another 2 h. The mixture was sequentially washed with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and water (10 ml), dried (Na₂SO₄), filtered and concentrated to give *N*-((2-methoxyphenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide. The crude product was used without any further purification. *N*-((2-Methoxyphenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide was dissolved in diethyl ether (2 ml) and HCl (1 ml, 4 M in dioxane) was added. The reaction mixture was stirred at room temperature. After 2.5 h, NaOH (1 ml, 6 N) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×15 ml), dried (Na₂SO₄), filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 40 mg; ¹³C-NMR (CD₃OD, rotamers): δ 26.1, 27.4, 40.0, 40.1, 43.5, 43.9, 51.5, 53.4, 54.5, 54.58, 54.63, 54.78, 54.83, 110.1, 110.5, 113.76, 113.78, 113.84, 114.0, 114.1, 120.1, 120.5, 125.4, 126.0, 126.5, 126.7, 127.1, 127.3, 127.7, 128.8, 129.8, 130.0, 130.08, 130.14, 156.5, 157.0, 159.0, 159.1, 173.2, 173.8.

Example 23 - *N*-((2-Chlorophenyl)methyl)-*N*-(piperidin-4-yl)-4-methoxyphenylacetamide (26HCH80-5)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (400 mg, 2 mmol) in methanol (1 ml) and 2-chlorobenzylamine (0.121 ml, 1 mmol) in methanol (1 ml) was added acetic acid in methanol (1 M, 1.34 ml) followed by NaCNBH₃ in methanol (0.3 M, 4.4 ml). The resulting solution was stirred at room temperature. After 24 h, water (2 ml) was added, and the mixture was stirred for 1 h before it was concentrated. The resulting oil was redissolved in diethyl ether (20 ml), extracted with HCl (0.1 N, 1×15 ml). The aqueous layer was washed with diethyl ether (10 ml) and treated with 0.2 N NaOH until basic (pH>8), before extracted with dichloromethane (20 ml). The organic layer was dried (Na₂SO₄),

filtered, and concentrated to give *tert*-butyl 4-((2-chlorophenyl)methyl)amino-piperidine carboxylate. Yield: 137 mg. To a solution of *tert*-butyl 4-((2-chlorophenyl)methyl)amino-piperidine carboxylate (50 mg, 0.15 mmol) in dichloromethane (6 ml) was added diisopropylethylamine (0.070 ml, 0.4 mmol) followed by 4-methoxyphenylacetyl chloride (0.055 ml, 0.35 mmol). The reaction mixture was stirred at room temperature. After 18 h, water (2 ml) was added. The mixture was stirred for another 2 h. The mixture was sequentially washed with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and water (10 ml), dried (Na₂SO₄), filtered and concentrated to give *N*-((2-chlorophenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide. The crude product was used without any further purification. *N*-((2-Chlorophenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide was dissolved in diethyl ether (2 ml) and HCl (1 ml, 4 M in dioxane) was added. The reaction mixture was stirred at room temperature. After 2.5 h, NaOH (1 ml, 6 N) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×15 ml), dried (Na₂SO₄), filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 45 mg; ¹³C-NMR (CD₃OD, rotamers): δ 25.8, 26.9, 40.0, 40.1, 42.9, 43.4, 43.7, 46.0, 51.1, 53.0, 54.6, 113.77, 113.84, 114.0, 114.1, 126.6, 126.8, 127.08, 127.13, 127.3, 127.4, 128.1, 129.0, 129.2, 129.8, 130.0, 130.2, 131.9, 132.2, 135.0, 135.3, 159.1, 173.4, 173.8.

Example 24 - *N*-((3,4-Di-methoxyphenyl)methyl)-*N*-(piperidin-4-yl)-4-methoxyphenylacetamide (26HCH80-6)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (400 mg, 2 mmol) in methanol (1 ml) and 3,4-di-methoxybenzylamine (0.151 ml, 1 mmol) in methanol (1 ml) was added acetic acid in methanol (1 M, 1.34 ml) followed by NaCNBH₃ in methanol (0.3 M, 4.4 ml). The resulting solution was stirred at room temperature. After 24 h, water (2 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. The resulting oil was redissolved in diethyl ether (20 ml), extracted with HCl (0.1 N, 1×15 ml). The aqueous layer was washed with diethyl ether (10 ml) and treated with 0.2 N NaOH until basic (pH>8), before

extracted with dichloromethane (20 ml). The organic layer was dried (Na_2SO_4), filtered, and concentrated to give *tert*-butyl 4-((3,4-di-methoxyphenyl)methyl)amino-piperidine carboxylate. Yield: 162 mg. To a solution of *tert*-butyl 4-((3,4-di-methoxyphenyl)methyl)amino-piperidine carboxylate (50 mg, 0.14 mmol) in dichloromethane (6 ml) was added diisopropylethylamine (0.070 ml, 0.4 mmol) followed by 4-methoxyphenylacetyl chloride (0.055 ml, 0.35 mmol). The reaction mixture was stirred at room temperature. After 18 h, water (2 ml) was added. The mixture was stirred for another 2 h. The mixture was sequentially washed with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and water (10 ml), dried (Na_2SO_4), filtered and concentrated to give *N*-((3,4-di-methoxyphenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide. The crude product was used without any further purification. *N*-((3,4-Di-methoxyphenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide was dissolved in diethyl ether (2 ml) and HCl (1 ml, 4 M in dioxane) was added. The reaction mixture was stirred at room temperature. After 2.5 h, NaOH (1 ml, 6 N) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×15 ml), dried (Na_2SO_4), filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH_3 in methanol, and concentrated to give the title compound. Yield: 54 mg; ^{13}C -NMR (CD_3OD , rotamers): δ 25.9, 27.3, 40.0, 40.1, 43.5, 43.8, 44.1, 51.4, 53.5, 54.6, 55.4, 110.2, 111.0, 111.9, 112.2, 114.0, 114.2, 118.6, 119.4, 127.1, 127.4, 129.9, 130.0, 130.5, 132.1, 148.2, 148.7, 149.2, 149.7, 158.98, 159.05, 173.3, 173.6.

Example 25 - *N*-((4-Fluorophenyl)methyl)-*N*-(piperidin-4-yl)-4-methoxyphenylacetamide (26HCH80-7)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (400 mg, 2 mmol) in methanol (1 ml) and 4-fluorobenzylamine (0.114 ml, 1 mmol) in methanol (1 ml) was added acetic acid in methanol (1 M, 1.34 ml) followed by NaCNBH_3 in methanol (0.3 M, 4.4 ml). The resulting solution was stirred at room temperature. After 24 h, water (2 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. The resulting oil was redissolved in diethyl ether (20 ml), extracted with HCl (0.1 N, 1×15 ml). The aqueous layer was washed

with diethyl ether (10 ml) and treated with 0.2 N NaOH until basic (pH>8), before extracted with dichloromethane (20 ml). The organic layer was dried (Na₂SO₄), filtered, and concentrated to give *tert*-butyl 4-((4-fluorophenyl)methyl)amino-piperidine carboxylate. Yield: 130 mg. To a solution of *tert*-butyl 4-((4-fluorophenyl)methyl)amino-piperidine carboxylate (50 mg, 0.16 mmol) in dichloromethane (6 ml) was added diisopropylethylamine (0.070 ml, 0.4 mmol) followed by 4-methoxyphenylacetyl chloride (0.055 ml, 0.35 mmol). The reaction mixture was stirred at room temperature. After 18 h, water (2 ml) was added. The mixture was stirred for another 2 h. The mixture was sequentially washed with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and water (10 ml), dried (Na₂SO₄), filtered and concentrated to give *N*-((4-fluorophenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide. The crude product was used without any further purification. *N*-((4-Fluorophenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide was dissolved in diethyl ether (2 ml) and HCl (1 ml, 4 M in dioxane) was added. The reaction mixture was stirred at room temperature. After 2.5 h, NaOH (1 ml, 6 N) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×15 ml), dried (Na₂SO₄), filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 45 mg; ¹³C-NMR (CD₃OD, rotamers): δ 26.1, 27.5, 40.1, 43.6, 43.8, 44.0, 51.6, 53.6, 54.6, 113.77, 113.84, 114.0, 114.1, 114.7, 114.9, 115.3, 115.6, 126.8, 127.2, 128.1, 128.6, 128.7, 129.8, 130.0, 130.1, 130.6, 131.0, 133.8, 159.1, 173.3, 173.6.

Example 26 - *N*-((2,4-Di-chlorophenyl)methyl)-*N*-(piperidin-4-yl)-4-methoxyphenylacetamide (26HCH80-8)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (400 mg, 2 mmol) in methanol (1 ml) and 2,4-di-chlorobenzylamine (0.135 ml, 1 mmol) in methanol (1 ml) was added acetic acid in methanol (1 M, 1.34 ml) followed by NaCNBH₃ in methanol (0.3 M, 4.4 ml). The resulting solution was stirred at room temperature. After 24 h, water (2 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. The resulting oil was redissolved in diethyl

ether (20 ml), extracted with HCl (0.1 N, 1×15 ml). The aqueous layer was washed with diethyl ether (10 ml) and treated with 0.2 N NaOH until basic (pH>8), before extracted with dichloromethane (20 ml). The organic layer was dried (Na₂SO₄), filtered, and concentrated to give *tert*-butyl 4-((2,4-di-chlorophenyl)methyl)amino-piperidine carboxylate. Yield: 97 mg. To a solution of *tert*-butyl 4-((2,4-di-chlorophenyl)methyl)amino-piperidine carboxylate (50 mg, 0.14 mmol) in dichloromethane (6 ml) was added diisopropylethylamine (0.070 ml, 0.4 mmol) followed by 4-methoxyphenylacetyl chloride (0.055 ml, 0.35 mmol). The reaction mixture was stirred at room temperature. After 18 h, water (2 ml) was added. The mixture was stirred for another 2 h. The mixture was sequentially washed with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and water (10 ml), dried (Na₂SO₄), filtered and concentrated to give *N*-((2,4-di-chlorophenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide. The crude product was used without any further purification. *N*-((2,4-Di-chlorophenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide was dissolved in diethyl ether (2 ml) and HCl (1 ml, 4 M in dioxane) was added. The reaction mixture was stirred at room temperature. After 2.5 h, NaOH (1 ml, 6 N) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×15 ml), dried (Na₂SO₄), filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 39 mg; ¹³C-NMR (CD₃OD, rotamers): δ 25.7, 26.8, 40.0, 42.6, 43.3, 43.7, 51.2, 53.0, 54.5, 54.6, 113.8, 113.8, 114.0, 114.1, 127.0, 128.4, 128.8, 129.8, 130.0, 130.1, 131.0, 132.7, 132.9, 134.5, 159.1, 173.4, 173.6.

Example 27 - *N*-((3-Methylphenyl)methyl)-*N*-(piperidin-4-yl)-4-methoxyphenylacetamide (26HCH80-9)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (400 mg, 2 mmol) in methanol (1 ml) and 3-methylbenzylamine (0.125 ml, 1 mmol) in methanol (1 ml) was added acetic acid in methanol (1 M, 1.34 ml) followed by NaCNBH₃ in methanol (0.3 M, 4.4 ml). The resulting solution was stirred at room temperature. After 24 h, water (2 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. The resulting oil was redissolved in diethyl

ether (20 ml), extracted with HCl (0.1 N, 1×15 ml). The aqueous layer was washed with diethyl ether (10 ml) and treated with 0.2 N NaOH until basic (pH>8), before extracted with dichloromethane (20 ml). The organic layer was dried (Na₂SO₄), filtered, and concentrated to give *tert*-butyl 4-((3-methylphenyl)methyl)amino-piperidine carboxylate. Yield: 136 mg. To a solution of *tert*-butyl 4-((3-methylphenyl)methyl)amino-piperidine carboxylate (50 mg, 0.16 mmol) in dichloromethane (6 ml) was added diisopropylethylamine (0.070 ml, 0.4 mmol) followed by 4-methoxyphenylacetyl chloride (0.055 ml, 0.35 mmol). The reaction mixture was stirred at room temperature. After 18 h, water (2 ml) was added. The mixture was stirred for another 2 h. The mixture was sequentially washed with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and water (10 ml), dried (Na₂SO₄), filtered and concentrated to give *N*-((3-methylphenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide. The crude product was used without any further purification. *N*-((3-Methylphenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide was dissolved in diethyl ether (2 ml) and HCl (1 ml, 4 M in dioxane) was added. The reaction mixture was stirred at room temperature. After 2.5 h, NaOH (1 ml, 6 N) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×15 ml), dried (Na₂SO₄), filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 48 mg; ¹³C-NMR (CD₃OD, rotamers): δ 20.4, 26.8, 28.3, 40.2, 43.9, 44.1, 44.5, 51.8, 54.2, 54.57, 54.61, 114.0, 114.1, 123.2, 123.7, 126.7, 127.0, 127.1, 127.3, 128.0, 128.1, 128.7, 129.8, 129.9, 137.9, 138.6, 138.9, 159.0, 159.1, 173.1, 173.7.

Example 28 - *N*-((3-Bromophenyl)methyl)-*N*-(piperidin-4-yl)-4-methoxyphenylacetamide (26HCH80-10)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (400 mg, 2 mmol) in methanol (1 ml) and 3-bromobenzylamine hydrobromide (222 mg, 1 mmol) in methanol (1 ml) was added acetic acid in methanol (1 M, 1.34 ml) followed by NaCNBH₃ in methanol (0.3 M, 4.4 ml). The resulting solution was stirred at room temperature. After 24 h, water (2 ml) was

added, and the mixture was stirred for 1 h, before it was concentrated. The resulting oil was redissolved in diethyl ether (20 ml), extracted with HCl (0.1 N, 1×15 ml). The aqueous layer was washed with diethyl ether (10 ml) and treated with 0.2 N NaOH until basic (pH>8), before extracted with dichloromethane (20 ml). The organic layer
5 was dried (Na₂SO₄), filtered, and concentrated to give *tert*-butyl 4-((3-bromophenyl)methyl)amino-piperidine carboxylate. Yield: 142 mg. To a solution of *tert*-butyl 4-((3-bromophenyl)methyl)amino-piperidine carboxylate (50 mg, 0.14 mmol) in dichloromethane (6 ml) was added diisopropylethylamine (0.070 ml, 0.4 mmol) followed by 4-methoxyphenylacetyl chloride (0.055 ml, 0.35 mmol). The
10 reaction mixture was stirred at room temperature. After 18 h, water (2 ml) was added. The mixture was stirred for another 2 h. The mixture was sequentially washed with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and water (10 ml), dried (Na₂SO₄), filtered and concentrated to give *N*-((3-bromophenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide. The crude product was
15 used without any further purification. *N*-((3-Bromophenyl)methyl)-*N*-(1-(*tert*-butyloxycarbonyl)piperidin-4-yl)-4-methoxyphenylacetamide was dissolved in diethyl ether (2 ml) and HCl (1 ml, 4 M in dioxane) was added. The reaction mixture was stirred at room temperature. After 2.5 h, NaOH (1 ml, 6 N) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×15 ml), dried
20 (Na₂SO₄), filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 49 mg; ¹³C-NMR (CD₃OD, rotamers): δ 26.6, 28.2, 40.2, 43.9, 44.0, 51.8, 54.1, 54.6, 113.76, 113.84, 114.1, 114.2, 122.2, 125.0, 125.5,
25 126.7, 127.1, 129.2, 129.5, 129.7, 129.8, 129.9, 130.0, 130.5, 130.6, 140.8, 141.8, 159.1, 173.3, 173.7.

Example 29 - *N*-(1-(Phenylmethyl)piperidin-4-yl)-*N*-(3-phenyl-2-propen-1-yl)-4-methoxyphenylacetamide (26HCH76B)

To a solution of 4-amino-*N*-benzylpiperidine (200 mg, 1.05 mmol) in
30 methanol (2 ml) was added trans-cinnamaldehyde (211 mg, 1.6 mmol), followed by Acetic acid in methanol (1 M, 1.4 ml) and sodiumcyanoborohydride in methanol (0.3 M, 4.4 ml). The reaction mixture was stirred at room temperature. After 48 h, water (2

ml) was added. The mixture was stirred for another 2 h before concentrated and redissolved in diethyl ether (20 ml). The organic layer was extracted with HCl (0.1 N, 2×10 ml). The combined aqueous layers were treated with NaOH (0.2 N) until basic (pH>8). The mixture was extracted with dichloromethane (2×10 ml). The combined
5 organic layers were dried (Na₂SO₄) and concentrated. The crude product, which was used without any further purification, was dissolved in dichloromethane (5 ml). Diisopropylethylamine (284 mg, 2.1 eq.) was added, followed by 4-methoxyphenylacetyl chloride (387 mg, 2.0 eq). The reaction mixture was stirred at room temperature. After 18 h, water (2 ml) was added. After additional 2 h
10 dichloromethane (10 ml) was added. The mixture was extracted with NaOH (0.2 N, 3×15 ml), and water (15 ml). The organic layer was dried (Na₂SO₄) and concentrated. The crude product was redissolved in methanol (2 ml) and added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and
15 concentrated to give the title compound. ¹³C-NMR (CDCl₃): δ 28.5, 38.1, 46.6, 47.4, 50.9, 54.7, 62.9, 113.7, 125.5, 126.4, 126.6, 127.4, 127.9, 128.5, 128.6, 129.6, 130.0, 135.2, 135.3, 138.0, 158.2, 173.2.

Example 30 - *N*-((4-Methylphenyl)methyl)-*N*-(1-piperidin-4-yl)-phenylacetamide (26HCH78-1)

20 To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (1.75 g, 8.8 mmol) and 4-methylbenzylamine (970 mg, 8.0 mmol) in methanol (7 ml) was added acetic acid in methanol (1 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 30 ml). The resulting solution was stirred at room temperature. After 20 h, water (5 ml) was added, and the mixture was stirred for 1 h,
25 before it was concentrated. Flash chromatography in dichloromethane:methanol 10:1 gave *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate. Yield: 2.4 g, 98%. To a solution of *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate (80 mg, 0.26 mmol) in dichloromethane (1.8 ml) was added diisopropylethylamine (0.11 ml, 2.4 eq.) followed by phenylacetyl chloride (81 mg,
30 0.53 mmol). The reaction mixture was stirred at room temperature. After 20 h, water (1 ml) was added. The mixture was stirred for another 2 h, before diethyl ether (20 ml) was added. The mixture was sequentially extracted with HCl (0.2 N, 2×15 ml),

NaOH (0.2 N, 2×15 ml), and H₂O (10 ml), dried (Na₂SO₄), filtered and concentrated. The crude material was dissolved in diethyl ether (2 ml) and HCl (4 M in dioxane, 1 ml). The reaction mixture was stirred at room temperature. After 2 h, NaOH (6 N, 1 ml) was added followed by dichloromethane (10 ml). The mixture was extracted with
5 water (2×10 ml), dried (Na₂SO₄), and filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 38 mg; ¹³C-NMR (CD₃OD, rotamers): δ 19.9, 26.9, 28.4, 41.0, 41.1, 44.0, 44.1, 44.4, 51.9, 54.4, 126.1,
10 126.7, 126.8, 126.9, 128.5, 128.7, 128.78, 128.81, 128.9, 129.4, 129.5, 134.9, 135.2, 135.6, 136.0, 136.3, 137.2, 172.8, 173.3.

Example 31 - N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-3-phenylpropionamide (26HCH78-2)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine
15 carboxylate (1.75 g, 8.8 mmol) and 4-methylbenzylamine (970 mg, 8.0 mmol) in methanol (7 ml) was added acetic acid in methanol (1 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 30 ml). The resulting solution was stirred at room temperature. After 20 h, water (5 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. Flash chromatography in dichloromethane:methanol 10:1
20 gave *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate. Yield: 2.4 g, 98%. To a solution of *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate (80 mg, 0.26 mmol) in dichloromethane (1.8 ml) was added diisopropylethylamine (0.11 ml, 2.4 eq.) followed by 3-phenylpropionyl chloride (0.078 ml, 0.53 mmol). The reaction mixture was stirred at room temperature. After
25 20 h, water (1 ml) was added. The mixture was stirred for another 2 h, before diethyl ether (20 ml) was added. The mixture was sequentially extracted with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and H₂O (10 ml), dried (Na₂SO₄), filtered and concentrated. The crude material was dissolved in diethyl ether (2 ml) and HCl (4 M in dioxane, 1 ml). The reaction mixture was stirred at room temperature. After 2 h,
30 NaOH (6 N, 1 ml) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×10 ml), dried (Na₂SO₄), and filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin

(0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 43 mg; ¹³C-NMR (CD₃OD, rotamers): δ 19.9, 27.4, 29.0, 31.4, 31.7, 34.7, 35.7, 44.2, 44.3, 51.6, 54.2, 125.9, 126.07, 126.15, 126.8, 128.3, 128.4, 128.7, 128.8, 129.3, 135.1, 136.1, 136.2, 137.0, 141.1, 141.2, 173.9, 174.4.

Example 32 - N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-(phenylthio)acetamide (26HCH78-3)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (1.75 g, 8.8 mmol) and 4-methylbenzylamine (970 mg, 8.0 mmol) in methanol (7 ml) was added acetic acid in methanol (1 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 30 ml). The resulting solution was stirred at room temperature. After 20 h, water (5 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. Flash chromatography in dichloromethane:methanol 10:1 gave *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate. Yield: 2.4 g, 98%. To a solution of *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate (80 mg, 0.26 mmol) in dichloromethane (1.8 ml) was added diisopropylethylamine (0.11 ml, 2.4 eq.) followed by (phenylthio)acetyl chloride (0.078 ml, 0.53 mmol). The reaction mixture was stirred at room temperature. After 20 h, water (1 ml) was added. The mixture was stirred for another 2 h, before diethyl ether (20 ml) was added. The mixture was sequentially extracted with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and H₂O (10 ml), dried (Na₂SO₄), filtered and concentrated. The crude material was dissolved in diethyl ether (2 ml) and HCl (4 M in dioxane, 1 ml). The reaction mixture was stirred at room temperature. After 2 h, NaOH (6 N, 1 ml) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×10 ml), dried (Na₂SO₄), and filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 18 mg; HRMS (FAB⁺, NBA) (M+H)⁺ 355.1841, C₂₁H₂₇N₂OS requires 355.1844; LC-MS: (M+H)⁺ 355.1, t_r 2.62 min.

Example 33 - N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-phenoxyacetamide (26HCH78-4)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (1.75 g, 8.8 mmol) and 4-methylbenzylamine (970 mg, 8.0 mmol) in methanol (7 ml) was added acetic acid in methanol (1 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 30 ml). The resulting solution was stirred at room temperature. After 20 h, water (5 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. Flash chromatography in dichloromethane:methanol 10:1 gave *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate. Yield: 2.4 g, 98%. To a solution of *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate (80 mg, 0.26 mmol) in dichloromethane (1.8 ml) was added diisopropylethylamine (0.11 ml, 2.4 eq.) followed by phenoxyacetyl chloride (0.073 ml, 0.53 mmol). The reaction mixture was stirred at room temperature. After 20 h, water (1 ml) was added. The mixture was stirred for another 2 h, before diethyl ether (20 ml) was added. The mixture was sequentially extracted with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and H₂O (10 ml), dried (Na₂SO₄), filtered and concentrated. The crude material was dissolved in diethyl ether (2 ml) and HCl (4 M in dioxane, 1 ml). The reaction mixture was stirred at room temperature. After 2 h, NaOH (6 N, 1 ml) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×10 ml), dried (Na₂SO₄), and filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 24 mg; ¹³C-NMR (CD₃OD, rotamers): δ 19.9, 25.8, 27.4, 43.5, 43.7, 44.4, 51.9, 52.3, 66.9, 114.7, 114.8, 116.7, 117.0, 121.4, 123.6, 126.3, 126.8, 128.4, 128.9, 129.3, 129.5, 129.6, 131.0, 134.4, 136.1, 137.4, 158.3, 169.8, 170.1.

Example 34 - N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-(4-chlorophenoxy)acetamide (26HCH78-5)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (1.75 g, 8.8 mmol) and 4-methylbenzylamine (970 mg, 8.0 mmol) in methanol (7 ml) was added acetic acid in methanol (1 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 30 ml). The resulting solution was stirred at room

temperature. After 20 h, water (5 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. Flash chromatography in dichloromethane:methanol 10:1 gave *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate. Yield: 2.4 g, 98%. To a solution of *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine
5 carboxylate (80 mg, 0.26 mmol) in dichloromethane (1.8 ml) was added diisopropylethylamine (0.11 ml, 2.4 eq.) followed by 4-chlorophenoxyacetyl chloride (0.082 ml, 0.53 mmol). The reaction mixture was stirred at room temperature. After 20 h, water (1 ml) was added. The mixture was stirred for another 2 h, before diethyl ether (20 ml) was added. The mixture was sequentially extracted with HCl (0.2 N,
10 2×15 ml), NaOH (0.2 N, 2×15 ml), and H₂O (10 ml), dried (Na₂SO₄), filtered and concentrated. The crude material was dissolved in diethyl ether (2 ml) and HCl (4 M in dioxane, 1 ml). The reaction mixture was stirred at room temperature. After 2 h, NaOH (6 N, 1 ml) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×10 ml), dried (Na₂SO₄), and filtered to give a clear solution.
15 The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 21 mg; ¹³C-NMR (CD₃OD, rotamers): δ 19.9, 26.2, 27.8, 43.6, 43.9, 44.4, 52.2, 52.5, 67.0, 116.2, 116.4, 126.2, 126.3, 126.8, 128.6, 128.9, 129.1, 129.3, 129.5, 131.0, 134.4,
20 135.6, 136.4, 137.5, 157.1, 169.4, 169.7.

Example 35 - N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-3-methoxyphenylacetamide (26HCH78-6)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (1.75 g, 8.8 mmol) and 4-methylbenzylamine (970 mg, 8.0 mmol) in
25 methanol (7 ml) was added acetic acid in methanol (1 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 30 ml). The resulting solution was stirred at room temperature. After 20 h, water (5 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. Flash chromatography in dichloromethane:methanol 10:1 gave *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate. Yield: 2.4 g,
30 98%. To a solution of *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate (80 mg, 0.26 mmol) in dichloromethane (1.8 ml) was added diisopropylethylamine (0.11 ml, 2.4 eq.) followed by 3-methoxyphenylacetyl chloride

(97 mg, 0.53 mmol). The reaction mixture was stirred at room temperature. After 20 h, water (1 ml) was added. The mixture was stirred for another 2 h, before diethyl ether (20 ml) was added. The mixture was sequentially extracted with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and H₂O (10 ml), dried (Na₂SO₄), filtered and concentrated. The crude material was dissolved in diethyl ether (2 ml) and HCl (4 M in dioxane, 1 ml). The reaction mixture was stirred at room temperature. After 2 h, NaOH (6 N, 1 ml) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×10 ml), dried (Na₂SO₄), and filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 26 mg; ¹³C-NMR (CD₃OD, rotamers): δ 19.9, 26.3, 27.7, 41.0, 43.7, 43.9, 44.4, 51.5, 53.8, 54.5, 54.6, 112.2, 112.6, 114.3, 114.5, 121.0, 121.2, 126.1, 126.8, 128.8, 129.4, 129.5, 129.8, 134.8, 136.0, 136.3, 136.5, 136.9, 137.2, 160.2, 160.3, 172.8, 173.2.

Example 36 - N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-4-fluorophenylacetamide (26HCH78-7)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (1.75 g, 8.8 mmol) and 4-methylbenzylamine (970 mg, 8.0 mmol) in methanol (7 ml) was added acetic acid in methanol (1 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 30 ml). The resulting solution was stirred at room temperature. After 20 h, water (5 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. Flash chromatography in dichloromethane:methanol 10:1 gave *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate. Yield: 2.4 g, 98%. To a solution of *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate (80 mg, 0.26 mmol) in dichloromethane (1.8 ml) was added diisopropylethylamine (0.11 ml, 2.4 eq.) followed by 4-fluorophenylacetyl chloride (0.072 ml, 0.53 mmol). The reaction mixture was stirred at room temperature. After 20 h, water (1 ml) was added. The mixture was stirred for another 2 h, before diethyl ether (20 ml) was added. The mixture was sequentially extracted with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and H₂O (10 ml), dried (Na₂SO₄), filtered and concentrated. The crude material was dissolved in diethyl ether (2 ml) and HCl (4 M in dioxane, 1 ml). The reaction mixture was stirred at room temperature. After 2 h,

NaOH (6 N, 1 ml) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×10 ml), dried (Na₂SO₄), and filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 26 mg; ¹³C-NMR (CD₃OD, rotamers): δ 19.9, 26.1, 27.4, 39.7, 39.9, 43.5, 43.8, 44.4, 51.3, 53.4, 114.9, 115.1, 115.3, 126.1, 126.7, 128.5, 128.8, 129.4, 130.7, 130.8, 130.9, 131.0, 131.2, 131.6, 134.8, 136.0, 136.3, 137.2, 160.9, 163.3, 172.7, 173.2.

Example 37 - N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-2,5-dimethoxyphenylacetamide (26HCH78-8)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (1.75 g, 8.8 mmol) and 4-methylbenzylamine (970 mg, 8.0 mmol) in methanol (7 ml) was added acetic acid in methanol (1 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 30 ml). The resulting solution was stirred at room temperature. After 20 h, water (5 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. Flash chromatography in dichloromethane:methanol 10:1 gave *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate. Yield: 2.4 g, 98%. To a solution of *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate (80 mg, 0.26 mmol) in dichloromethane (1.8 ml) was added diisopropylethylamine (0.11 ml, 2.4 eq.) followed by 2,5-di-methoxyphenylacetyl chloride (0.092 ml, 0.53 mmol). The reaction mixture was stirred at room temperature. After 20 h, water (1 ml) was added. The mixture was stirred for another 2 h, before diethyl ether (20 ml) was added. The mixture was sequentially extracted with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and H₂O (10 ml), dried (Na₂SO₄), filtered and concentrated. The crude material was dissolved in diethyl ether (2 ml) and HCl (4 M in dioxane, 1 ml). The reaction mixture was stirred at room temperature. After 2 h, NaOH (6 N, 1 ml) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×10 ml), dried (Na₂SO₄), and filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. 36 mg; ¹³C-NMR (CD₃OD, rotamers): δ 20.0, 26.5, 28.2, 35.1,

35.7, 44.0, 44.4, 51.6, 53.8, 54.99, 55.03, 55.2, 55.5, 111.4, 111.7, 112.4, 112.9, 116.6, 116.9, 124.98, 125.02, 126.1, 126.7, 128.8, 129.3, 135.0, 136.1, 136.3, 137.0, 151.3, 151.7, 153.9, 154.0, 173.1, 173.5.

Example 38 - N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-4-chlorophenylacetamide (26HCH78-9)

To a solution of commercially available *tert*-butyl 4-oxo-1-piperidine carboxylate (1.75 g, 8.8 mmol) and 4-methylbenzylamine (970 mg, 8.0 mmol) in methanol (7 ml) was added acetic acid in methanol (1 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 30 ml). The resulting solution was stirred at room temperature. After 20 h, water (5 ml) was added, and the mixture was stirred for 1 h, before it was concentrated. Flash chromatography in dichloromethane:methanol 10:1 gave *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate. Yield: 2.4 g, 98%. To a solution of *tert*-butyl 4-(4-methylphenyl)methylamino-piperidine carboxylate (80 mg, 0.26 mmol) in dichloromethane (1.8 ml) was added diisopropylethylamine (0.11 ml, 2.4 eq.) followed by 4-chlorophenylacetyl chloride (99 mg, 0.53 mmol). The reaction mixture was stirred at room temperature. After 20 h, water (1 ml) was added. The mixture was stirred for another 2 h, before diethyl ether (20 ml) was added. The mixture was sequentially extracted with HCl (0.2 N, 2×15 ml), NaOH (0.2 N, 2×15 ml), and H₂O (10 ml), dried (Na₂SO₄), filtered and concentrated. The crude material was dissolved in diethyl ether (2 ml) and HCl (4 M in dioxane, 1 ml). The reaction mixture was stirred at room temperature. After 2 h, NaOH (6 N, 1 ml) was added followed by dichloromethane (10 ml). The mixture was extracted with water (2×10 ml), dried (Na₂SO₄), and filtered to give a clear solution. The solution was added on to a column carrying strongly acidic cation exchange resin (0.3 mmol/g resin), which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and concentrated to give the title compound. Yield: 22 mg; ¹³C-NMR (CD₃OD, rotamers): δ 19.9, 26.3, 27.7, 39.9, 40.0, 43.6, 43.9, 44.4, 51.5, 53.6, 126.1, 126.7, 128.2, 128.4, 128.6, 128.9, 129.4, 129.6, 130.7, 130.9, 131.2, 131.6, 132.5, 132.7, 133.9, 134.1, 134.4, 134.8, 135.9, 136.3, 137.2, 172.4, 172.9.

Example 39 - *N*-((4-Methylphenyl)methyl)-*N*-(1-(phenylmethyl)pyrrolidin-3-yl)-*N*³-phenylmethylcarbamide (26HCH50)

To a solution of 3-amino-1-phenylmethylpyrrolidine (353 mg, 2 mmol) and 4-methylbenzaldehyde (361 mg, 3 mmol) in methanol (20 ml) was added acetic acid in
5 methanol (2 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 3 ml). The mixture was stirred at room temperature. After 24 h, water (5 ml) was added. The mixture was stirred for another hour before concentrated. Flash chromatography in dichloromethane/methanol 10/1 gave *N*-((4-methylphenyl)methyl)amino-1-phenylmethylpyrrolidine.

10 *N*-((4-Methylphenyl)methyl)amino-1-phenylmethylpyrrolidine (35 mg, 0.125 mmol) was dissolved in dichloromethane (1.5 ml), and benzylisocyanate (0.09 ml, 0.3 mmol) was added. The reaction mixture was stirred at room temperature. After 48 h, the crude reaction mixture was added on to a column carrying strongly acidic cation exchange resin, which was washed with methanol (3×6 ml), and eluted with 10%
15 NH₃ in methanol, and concentrated to give the title compound. Yield: 48 mg, 92%;
¹³C-NMR (CD₃OD): δ 20.0, 29.7, 44.2, 51.3, 53.4, 56.4, 57.8, 58.7, 126.8, 127.1, 127.3, 127.6, 128.3, 128.4, 128.9, 129.1, 135.9, 136.8, 140.3, 158.5.

Example 40 - *N*-((4-Methylphenyl)methyl)-*N*-(1-(phenylmethyl)pyrrolidin-3-yl)-4-methoxyphenylacetamide (26HCH52)

20 To a solution of 3-amino-1-phenylmethylpyrrolidine (353 mg, 2 mmol) and 4-methylbenzaldehyde (361 mg, 3 mmol) in methanol (20 ml) was added acetic acid in methanol (2 M, 6.7 ml) followed by NaCNBH₃ in methanol (0.3 M, 3 ml). The mixture was stirred at room temperature. After 24 h, water (5 ml) was added. The mixture was stirred for another hour before concentrated. Flash chromatography in
25 dichloromethane/methanol 10/1 gave *N*-((4-methylphenyl)methyl)amino-1-phenylmethylpyrrolidine.

To a solution of *N*-((4-Methylphenyl)methyl)amino-1-phenylmethylpyrrolidine (35 mg, 0.125 mmol), diisopropylethylamine (0.14 ml) in dichloromethane (1.5 ml) was added 4-methoxyphenylacetyl chloride (0.1 ml, 0.5
30 mmol). The reaction mixture was stirred at room temperature. After 48 h, the crude reaction mixture was concentrated and redissolved in methanol. The solution was added on to a column carrying strongly acidic cation exchange resin, which was washed with methanol (3×6 ml), and eluted with 10% NH₃ in methanol, and

concentrated. Flash chromatography in dichloromethane/methanol 10/1 gave the title compound. Yield: 20 mg, 38%; ^{13}C -NMR (CD_3OD): δ 21.3, 30.2, 40.8, 47.8, 53.6, 53.9, 55.5, 57.5, 60.2, 114.4, 125.7, 127.0, 127.1, 127.3, 127.4, 128.4, 128.5, 128.7, 128.9, 129.2, 129.8, 130.0, 135.9, 137.0, 158.6.

5 **Example 41 - N-((4-Methylphenyl)methyl)-N-(1-(phenylmethyl)piperidin-4-yl)-4-methoxyphenylthioacetamide (RO)**

A mixture of *N*-((4-Methylphenyl)methyl)-*N*-(1-(phenylmethyl)piperidin-4-yl)-(4-methoxyphenylmethyl) acetamide (20 mg, 0.045mmol) and Lawesson's reagent (25 mg, 0.062 mmol), was taken in a glass vial and mixed thoroughly with
10 magnetic stirbars. The glass vial was then irradiated in a microwave oven (900 W, Whirlpool M401) for 8 min. Upon completion of the reaction, the yellow-colored material was transferred to an ion-exchange column with the aid of methanol (2 ml). The ion-exchange column was subsequently washed with CH_2Cl_2 (2ml) and methanol (2ml) and the product was thereafter eluted from the ion-exchange column (10% NH_3
15 in methanol, 2 ml) to give *N*-((4-Methylphenyl)methyl)-*N*-(1-(phenylmethyl)piperidin-4-yl)-4-methoxyphenylmethyl thioacetamide (20 mg, 97%) as a white solid; LC-MS: $(\text{M}+\text{H})^+$ 459, t_r 9.60 min; TLC (CH_2Cl_2 /methanol 20:1) R_f = 0.38.

Example 42 - Receptor Selection and Amplification (R-SAT) Assays.

20 The functional receptor assay, Receptor Selection and Amplification Technology (R-SAT), was used (with minor modifications from that previously described US 5,707,798) to screen compounds for efficacy at the 5-HT_{2A} receptor. Briefly, NIH3T3 cells were grown in 96 well tissue culture plates to 70-80% confluence. Cells were transfected for 12-16 hours with plasmid DNAs using
25 superfect (Qiagen Inc.) as per manufacture's protocols. R-SAT's were generally performed with 50 ng/well of receptor and 20 ng/well of Beta-galactosidase plasmid DNA. All receptor and G-protein constructs used were in the pSI mammalian expression vector (Promega Inc) as described in U.S. 5,707,798. The 5HT_{2A} receptor gene was amplified by nested PCR from brain cDNA using the oligodeoxynucleotides
30 based on the published sequence (see Saltzman et. al. *Biochem. Biophys. Res. Comm.* 181:1469-78 (1991)). Large-scale transfections, cells were transfected for 12-16 hours, then trypsinized and frozen in DMSO. Frozen cells were later thawed, plated at 10,000-40,000 cells per well of a 96 well plate that contained drug. With both

methods, cells were then grown in a humidified atmosphere with 5% ambient CO₂ for five days. Media was then removed from the plates and marker gene activity was measured by the addition of the beta-galactosidase substrate ONPG (in PBS with 5% NP-40). The resulting colorimetric reaction was measured in a spectrophotometric plate reader (Titertek Inc.) at 420 nM. All data were analyzed using the computer program XLFit (IDBSm). Efficacy is the percent maximal repression compared to repression by a control compound (ritanserine in the case of 5HT_{2A}). pIC₅₀ is the negative of the log(IC₅₀), where IC₅₀ is the calculated concentration in Molar that produces 50% maximal repression. The results obtained for six compounds of the invention are presented in the following table.

Table 1. Efficacy of Compounds at the 5-HT_{2A} Receptor

Compound	Efficacy (average)	Efficacy(s tdev)	pIC ₅₀ (average)	pIC ₅₀ (stdev)
26HCH52	98	5.0	7.31	0.16
26HCH66-03	76	13.3	7.42	0.01
26HCH66-05	109	3.0	7.55	0.15
26HCH80-2	89	4.6	7.78	0.17
26HCH80-7	87	3.7	7.70	0.26
26HCH80-10	91	4.9	7.21	0.05

Example 43 - In Vitro Efficacy of 26HCH17 as an Inverse Agonist at the 5-HT_{2A} Receptor.

The graph shown in Figure 1 represents the data obtained from a dose response analysis of 26HCH17 and ritanserine as 5-HT_{2A} receptor inverse agonists. Briefly, the 5-HT_{2A} receptor, and the alpha subunit of the guanine nucleotide binding protein Gq were transiently transfected into NIH3T3 cells and assayed using the functional receptor assay, Receptor Selection and Amplification Technology (R-SAT) essentially as disclosed in U.S. Patent No. 5,707,798. Each compound was screened at seven serially diluted concentrations in triplicate. Data were analyzed using least squares fit analysis with GraphPad Prism (San Diego, CA.), and are reported normalized to percent response.

Example 44 - Selectivity Profile of Inverse Agonist 26HCH16D

R-SAT assays (as described in Example 42) were carried out with cells transfected with receptors (listed below) to determine the receptor selectivity profile for compound 26HCH16D. 5HT_{2A} inverse agonist data (IC₅₀ nM; % efficacy) were

derived from detailed dose response curves (7 points in triplicate). All other data (initial concentration at which at least 30% efficacy observed; actual efficacy figure) derived from the 4 dose profiling protocol in which compounds were tested at 4 doses in duplicate. nr = activity less than 30% at all doses tested (3, 30, 300, 3000nM), therefore EC₅₀/IC₅₀ greater than 3000nM). The results are presented in the following table.

Table 2. Profile of 5-HT_{2A} Inverse Agonist 26HCH16D

Receptor		Efficacy
5HT _{2A} (human)	Agonist	nr
	Inverse Agonist	0.9nM; 79%
5HT _{2B} (human)	Agonist	nr
	Antagonist	3000nM; 60%
5HT _{2C} (human)	Agonist	nr
	Inverse Agonist	3000nM; 79%
5HT _{1A} (human)	Agonist	nr
	Antagonist	nr
5HT _{1A} (rat)	Antagonist	nr
5HT _{1E} (human)	Agonist	nr
D ₂ (human)	Agonist	nr
	Antagonist	3000nM; 73%
H ₁ (human)	Agonist	nr
	Antagonist	3000nM; 30%
alpha _{1a/D} (rat)	Agonist	nr
	Antagonist	nr
alpha _{1b/B} (hamster)	Agonist	nr
	Antagonist	nr
alpha _{1c/A} (human)	Agonist	nr
	Antagonist	3000nM; 46%
alpha _{2A} (human)	Agonist	nr
	Antagonist	nr
alpha _{2B} (human)	Agonist	nr
	Antagonist	nr
alpha _{2C} (human)	Agonist	nr
	Antagonist	nr
m ₁ (human)	Agonist	nr
	Antagonist	nr

As indicated above, 26HCH16D is a highly selective 5-HT_{2A} inverse agonist.

General LC-MS procedure for Working Examples ELH01-46, MBT01-14 and AKU01-38.

In the following examples, HPLC/MS analyses were performed using either of two general methods (Method A or Method B). The t_r values reported below were
5 obtained using one of these procedures, as indicated in the specific examples.

The methods were as follows:

Method A: Agilent HP1100 HPLC/MSD.

G1312A Binary pump, G1313A Autosampler, G1316A Column compartment, G1315A Diode array detector (190-450 nm), 1946A MSD, electrospray ionization.

10 *Chromatography:*

8 mM ammoniumacetate in water/acetonitrile.

Gradient start at 70% org. up to 100 % org. over 12 min, down to 70 % org. over 0.5 min, held for 3.5 min. Total runtime 16 min. Flowrate 1 ml/min

Column, Phenomenex Luna C18(2) 3 μ m 75x4.6mm.

15 *MS parameters:*

Drying gas, 10 l/min. Nebulizer pressure, 40 psig. Gas temp, 350 C. VCap, 4000.

Method B: Waters/Micromass HPLC/MS

600 LC-pump, 2700 Sample manager, 2487 Dual absorbance detector
20 (channel A-205 nm, channel B-235 nm), Micromass ZMD-mass-spectrometer, electrospray ionization.

Chromatography:

0.15%TFA in water/acetonitrile.

Gradient start at 30 % org. up to 100% org. over 10 min, held for 3 min. down
25 to 30 % org. over 0.5 min, held for 4.5 min. Total run time 18 min. Flowrate, 1 ml/min.

Column, Symmetry C18, 5 μ m, 4.6x50 mm. or

10 mM ammoniumacetate in water/acetonitrile.

Gradient start at 30% org. for 2.5 min, up to 100 % org. over 10 min, held for
30 9 min, down to 30 % org. over 0.5 min, held for 5 min. Total run time 27 min. Flowrate, 1 ml/min.

Column: Phenomenex Synergi C12, 4 μ m, 4.6x50mm.

MS parameters:

Desolvation gas, 404 l/H. Capillary, 5.3kV. Cone, 36V. Extractor, 3V.
Source block temp, 130 C. Desolvation temp, 250 C.

Example 45 - 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(piperidin-4-yl) acetamide (50ELH87)

5 *Reaction step 1: N-trifluoroacetyl-4-piperidone (50ELH84)*

4-Piperidone hydrochloride monohydrate (4.0 g, 26 mmol, 1.0 eq) was dissolved in 130 ml of dichloromethane. After addition of triethylamine (8.66 g, 3.3 eq) the reaction mixture was stirred for 10 min. The mixture was cooled on an ice-bath (0°C). Trifluoroacetic anhydride (12.0 g, 2.2 eq) was added dropwise under
10 stirring. After 2 hours the reaction was quenched by addition of distilled water. The aqueous phase was extracted twice with dichloromethane. The combined organic layers were collected and dried with sodium sulfate. Concentration afforded *N*-trifluoroacetyl-4-piperidone.

Reaction step 2: 4-(4-Methylbenzylamino)-1-(trifluoroacetyl) piperidin (50ELH85)

15 Methanol (150 ml) was added to an Erlenmeyer flask and acetic acid was added under stirring until pH 5. 4-Methylbenzylamine (3.14 g, 25.9 mmol) and *N*-trifluoroacetyl-4-piperidone (from reaction step 1) (5.065 g, 25.9 mmol) were added to a 250 ml round-bottomed flask and dissolved in the methanol/acetic acid (150 ml) solution previously made. The reaction mixture was stirred for 5 min and NaCNBH₃
20 (2.46 g, 38.9 mmol) was added slowly under stirring. After 20 hours the reaction was concentrated and transferred to a separatory funnel containing dichloromethane and distilled water. The aqueous phase was made basic by addition of Na₂CO₃. The aqueous phase was extracted twice with dichloromethane. The combined organic layers were collected and dried with Na₂SO₄. Concentration afforded, 4-(4-methylbenzylamine)-1-(trifluoroacetyl) piperidine. UV/MS 60/53 (M⁺ 301), t_r (A, MS) 3.267.

Reaction step 3: 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-trifluoroacetyl)piperidin-4-yl) acetamide (50ELH86)

30 The product from reaction step 2 (7.8 g, 25.9 mmol) was dissolved in 100 ml of dichloromethane and stirred while 4-methoxyphenylacetyl chloride (4.8 g, 25.9 mmol) was added. After 4 hours, heptane was added whereupon the product precipitated as the hydrochloride salt. The solvent was removed by evaporation. The crude material was purified by flash chromatography EtOAc/Heptane (1:2) Yield (overall: Reaction steps 1+2+3) 3.912 g (34%), UV/MS 91/58 (M⁺ 449), t_r (A, MS)

4.319. ¹H-NMR (400 MHz, CDCl₃) δ 6.80-7.15 (Ar, 4H), 4.64 (brt, 1H), 4.4 (s, 2H), 3.95 (d, 2H), 3.72 (s, 3H), 3.50 (s, 2H), 3.09 (t, 2H), 2.7 (t, 2H), 2.32 (s, 3H), 1.75 (brt, 2H). ¹³C-NMR 172.5; 158.8; 137.4; 134.9; 129.9; 129.9; 129.8; 127.1; 125.8; 114.3; 55.4; 52.2; 47.3; 45.3; 43.4. 40.6; 30.1; 29.2; 21.2.

5 *Reaction step 4: 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(piperidin-4-yl) acetamide (50ELH87)*

The product from reaction step 3 (3.9 g, 8.7 mmol) was dissolved in methanol (12 ml). In a 250 ml round bottom flask a saturated solution of potassium carbonate in methanol was prepared. To this solution, the 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(N-trifluoroacetyl-
10 piperidin-4-yl) acetamide solution was added under stirring. After 4 hours, the solution was concentrated and the remaining solid taken up in base and dichloromethane. The combined organic layers were dried with sodium sulfate and concentrated. UV/MS 91/72 (M⁺ 353), t_r (A, MS) 2.210.

The corresponding hydrochloride salt was also prepared, by dissolving the free
15 base in dichloromethane (1 ml) and HCl (1 eq. 2 M HCl in ether) was added with stirring. The salt was precipitated by addition of the dichloromethane solution into heptane. Concentration on the rotary evaporator returned the product as white crystals.

Example 46 - 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH27)

20 *Reaction step 1: 4-(4-Methylbenzylamino)-1-methylpiperidine (50ELH25)*

Methanol (50 ml) was added to an Erlenmeyer flask and acetic acid was added under stirring until pH 5. Methylbenzylamine (1.0 g, 8.8 mmol) and 1-Methyl-4-piperidone (1.1 g, 8.8 mmol) were added to a 100 ml round-bottomed flask and dissolved in the methanol/acetic acid (40 ml) solution previously made. The reaction
25 mixture was stirred for 5 min and NaCNBH₃ (0.83 g, 13.2 mmol) was added slowly under stirring. After 20 hours the reaction was concentrated and transferred to a separatory funnel containing dichloromethane and distilled water. The aqueous phase was made basic by addition of Na₂CO₃. The aqueous phase was extracted twice with dichloromethane. The combined organic layers were collected and dried with Na₂SO₄.
30 Concentration afforded the title compound. Yield (crude): 98%. UV/MS 89/88 (M⁺ 353), t_r (A, MS) 3.982.

Reaction step 2: 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH27)

The product from reaction step 1 (1.9 g, 8.7 mmol) was dissolved in 40 ml of dichloromethane and stirred while 4-methoxyphenylacetylchloride (1.606 g, 8.7 mmol) was added. After 4 hours, heptane was added whereupon the product precipitated as the hydrochloride salt. The solvent was removed by evaporation. The crude material was purified by flash chromatography first eluting with 10% MeOH in CH₂Cl₂ and thereafter eluting with 0-20% MeOH in CH₂Cl₂ and 5% NEt₃. Yield (overall: Reaction steps 1+2): 77%. UV/MS: 100/100 (M⁺ 367), t_r (A, MS) 4.359, R_f 0.15 (2% MeOH in CH₂Cl₂). ¹H-NMR (400 MHz, CDCl₃) δ 12.6 (s, 1H), 7.16 (d, J=7.0 Hz, 2H), 7.10 (d, J=7.0 Hz, 2H), 7.04 (d, J=8.0 Hz, 2H), 6.82 (d, J=8.0 Hz, 2H), 4.87 (tt, J=11.0, 4.0 Hz, 1H), 4.53 ppm (s, 2H), 3.78 (s, 3H), 3.55 (s, 2H), 3.42 (brd, J=11.0 Hz, 2H), 2.80 (brq, J=11.0 Hz, 2H), 2.7 (d, J=4.0 Hz, 3H), 2.42 (dq, J=13.0, 3.0 Hz, 2H), 2.34 (s, 3H), 1.78 (brd, J=13.0 Hz, 2H). ¹³C-NMR 173.1; 158.9; 137.4; 134.8; 129.9; 126.7; 125.8; 114.4; 76.9; 55.5; 54.6; 48.8; 43.7; 40.5; 26.4; 21.2

Example 47 - 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-cyclohexylmethylpiperidin-4-yl) acetamide (42ELH45)

50ELH87 (the hydrochloride salt) (0.5 g, 1.29 mmol, 1.0 eq) was dissolved in ethanol (100 ml). Cyclohexanecarboxaldehyde (2.5 g, 20 eq.) was added followed by addition of sodium borohydride (0.084 g, 2.0 eq.). The reaction was stirred for 36 h and acetic acid (3 ml) was added. The reaction was stirred for additionally 2 h and extracted with sodium hydrogen carbonate (3 times) and dichloromethane. The organic layers were dried with sodium sulfate and concentrated. The product was purified by flash chromatography (1-10% MeOH in CH₂Cl₂). The resulting product was dissolved in ether (20 ml) and MeOH (added dropwise until dissolved) and HCl (1 eq. 2 M HCl in ether) was added under stirring. The hydrochloride salt of 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(1-cyclohexylmethylpiperidin-4-yl) acetamide precipitated and the white crystals were filtered. Yield 80 mg (16%), UV/MS 100/100 (M⁺ 449), t_r (A, MS) 7.105, mp 133-135°C, R_f 0.25 (2% MeOH/CH₂Cl₂). ¹H-NMR (400 MHz, CDCl₃) δ 11.9 (brs, 1H), 7.12 (q, 4H), 7.02 (d, 2H), 6.80 (d, 2H), 4.87 (m, 1H), 4.58 (s, 2H), 3.77 (s, 3H), 3.55 (s, 2H), 3.48 (m, 2H), 2.70 (m, 4H), 2.31 (s, 3H), 1.91 (d, 2H), 1.75 (m, 3H), 1.64 (d, 1H), 1.22 (d, 2H), 1.13 (tt, 2H), 1.02 (brq, 2H). ¹³C-NMR 173.1; 158.8; 137.2; 135.1; 129.9; 129.8; 126.8; 125.8; 114.4; 64.1; 55.5; 53.4; 49.2; 46.5; 40.4; 33.9; 25.9; 25.8; 25.7; 21.2.

Example 48 - 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-ethylpiperidin-4-yl) acetamide (42ELH80)

50ELH87 (0.25 g, 0.71 mmol, 1.0 eq) was dissolved in acetonitrile (15 ml) and ethyl bromide (0.232 g, 3.0 eq.) was added under stirring. After 2 min Hünings
5 base (0.084 g, 10.0 eq.) was added. After 36 hours, the solution was extracted with sodium hydrogen carbonate solution and dichloromethane (3 times). The organic layers were dried with sodium sulfate and concentrated yielding a yellow oil. The product was purified by flash chromatography (2% MeOH in CH₂Cl₂). The resulting product was dissolved in dichloromethane (1 ml) and HCl (1 eq. 2 M HCl in ether)
10 was added under stirring. The salt was precipitated by addition of the dichloromethane solution into heptane. Concentration on the rotary evaporator gave the product as white crystals. Yield 170 mg (63%), UV/MS 98/95 (M⁺ 381), mp 153-155°C, τ_f (A, MS) 3.033, R_f 0.35 (3% MeOH/CH₂Cl₂). ¹H-NMR (400 MHz, CDCl₃) δ 12.2 (s, 1H), 7.15 (d, 2H), 7.12 (d, 2H), 7.08 (d, 2H), 6.82 (d, 2H), 4.89 (m, 1H), 4.58
15 (s, 2H), 3.79 (s, 3H), 3.58 (s, 2H), 3.50 (d, 2H), 2.90 (m, 1H), 2.7 (brq, 2H), 2.45 (m, 2H), 2.34 (s, 3H), 1.80 (d, 2H), 1.44 (t, 3H). ¹³C-NMR 173.1; 158.9; 137.3; 134.9; 129.9; 125.8; 114.4; 55.5; 52.3; 52.0; 49.2; 46.5; 40.5; 26.2; 21.2; 9.5.

Example 49 - 2-(4-Methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-ethylpiperidin-4-yl) acetamide (42ELH85).

20 This compound was prepared similarly to 50ELH27

Reaction-step 1: (42ELH84)

Starting materials: 1-Methyl-4-piperidone (0.5 g, 4.4 mmol, 1.0 eq.), 4-chlorobenzylamine (0.626 g, 1.0 eq.), sodium cyanoborohydride (0.279 g, 1.5 eq.).

Reaction-step 2: (42ELH85)

25 *Starting materials:* 42ELH84, 4-methoxyphenylacetylchloride (0.774 g, 1.0 eq.).

The procedure was analogous to 50ELH27, but the product was purified by ion-exchange chromatography followed by HPLC. The hydrochloride salt was made by dissolving the free base in dichloromethane (1 ml) and HCl (1 eq. 2 M HCl in
30 ether) was added under stirring. The salt was precipitated by addition of the dichloromethane solution into heptane followed by concentration on the rotary evaporator.

Product: White crystals. UV/MS 98/97 (M^+ 387), r_t (A, MS) 2.953. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 12.6 (s, 1H), 7.35 (d, 2H), 7.18 (d, 2H), 7.05 (d, 2H), 6.82 (d, 2H), 4.89 (m, 1H), 4.55 (s, 2H), 3.80 (s, 3H), 3.55 (s, 2H), 3.45 (brs, 2H), 2.80 (brs, 2H), 2.72 (s, 3H), 2.25 (brs, 3H), 1.80 (brs, 2H). $^{13}\text{C-NMR}$ 173.0; 158.9; 136.5; 133.6; 129.8; 129.4; 127.3; 126.3; 114.5; 55.5; 54.6; 48.7; 46.3; 43.7; 40.5; 26.3.

50 - 2-(4-Methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-isopropylpiperidin-4-yl) acetamide (42ELH79).

Procedure as 42ELH80

Starting materials: 50ELH87 (0.25 g, 0.71 mmol, 1.0 eq.),
10 isopropylbromide (0.262 g, 3.0 eq.).

Product: Yield 130 mg (46%), UV/MS 100/100 (M^+ 395), r_t (A, MS) 3.360. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 12.0 (s, 1H), 7.15 (d, 2H), 7.10 (d, 2H), 7.05 (d, 2H), 6.82 (d, 2H), 4.87 (m, 1H), 4.60 (s, 2H), 3.79 (s, 3H), 3.57 (s, 2H), 3.38 (brd, 3H), 2.79 (q, 2H), 2.63 (q, 2H), 2.34 (s, 3H), 1.80 (d, 2H), 1.39 (d, 6H). $^{13}\text{C-NMR}$ 173.1; 158.9; 137.3; 135.1; 129.8; 126.8; 125.8; 114.4; 57.9; 49.4; 48.2; 46.5; 40.5; 25.9; 21.2; 16.9.

Example 51 - 2-(4-Methoxyphenyl)-N-(4-chlorobenzyl)-N-(piperidin-4-yl) acetamide (42ELH89) (As starting material in other reactions, used unpurified)

Procedure as 50ELH27.

20 Reaction step 1: N-Trifluoroacetyl-4-piperidone (42ELH86)

Starting materials: 4-Piperidone hydrochloride monohydrate (2.0 g, 13 mmol, 1.0 eq), trifluoroacetic anhydride (6.0, 2.2 eq.). TLC showed full conversion.

Product: R_f 0.9 (10% MeOH/ CH_2Cl_2).

Reaction step 2: 4-(4-Chlorobenzylamino)-1-(trifluoroacetyl) piperidin
25 (42ELH87)

Starting materials: 42ELH86 (2.5 g, 12.8 mmol, 1.0 eq.), 4-Chlorobenzylamine (1.8 g, 1.0 eq.)

Reaction step 3: 2-(4-Methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-trifluoroacetyl)piperidin-4-yl) acetamide (42ELH88)

30 *Starting materials:* 42ELH87 (4.0 g, 12.5 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (2.31 g, 1.0 eq.)

Reaction step 4: 2-(4-Methoxyphenyl)-N-(4-chlorobenzyl)-N-(piperidin-4-yl) acetamide (42ELH89)

Product: Yield: 2 g (57%), UV/MS 80/82 (M^+ 373), R_f 0.2 (50% EtOAc/Heptane).

Example 52 - 2-(4-Methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-cyclopentylpiperidin-4-yl) acetamide (42ELH91).

5 Procedure as 42ELH80, but the product was purified by HPLC. The acidic eluent was made basic with sodium carbonate and extracted with dichloromethane (3 times). The combined organic layers were collected and dried with sodium sulfate and concentrated. The remaining product was dissolved in 1 ml of dichloromethane and HCl (1 eq. 2 M HCl in ether) was added under stirring. This solution was added drop-
10 wise to a large excess of n-heptane to make the hydrochloride precipitate. The solvent was evaporated off to form white crystals of 2-(4-methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-cyclopentylpiperidin-4-yl) acetamide, hydrochloride.

Starting materials: 42ELH89 (0.25 g, 0.67 mmol, 1.0 eq.), cyclopentylbromide (0.3, 3.0 eq.)

15 *Product:* Yield: 211.2 mg (76%). Purification by ion-exchange: UV/MS 90/98. Purification by HPLC UV/MS 100/100 (M^+ 441), R_f 0.2 (3% MeOH/ CH_2Cl_2), r_f (A, MS) 4.067. 1H -NMR (400 MHz, $CDCl_3$) δ 12.2 (brs, 1H), 7.32 (d, 2H), 7.17 (d, 2H), 7.04 (d, 2H), 6.82 (d, 2H), 4.90 (brt, 1H), 4.58 (s, 2H), 3.79 (s, 3H), 3.58 (brd, 2H), 3.54 (s, 2H), 3.14 (brq, 2H), 2.58 (brq, 2H), 2.04 (m, 4H), 1.89 (m, 4H), 1.75 (brd, 20 2H). ^{13}C -NMR 173.0; 158.9; 133.5; 129.8; 129.3; 127.3; 126.4; 114.5; 68.4; 55.5; 51.9; 49.1; 46.2; 40.5; 28.5; 26.0; 23.8.

Example 53 - 2-(4-Methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-isopropylpiperidin-4-yl) acetamide (42ELH90).

42ELH89 (0.25 g, 0.67 mmol, 1.0 eq) was transferred to a 4 ml vial and
25 dissolved in acetonitrile (2 ml). Isopropyl bromide (0.25 g, 3.0 eq.) was added along with Hünigs base (0.87 g, 10.0 eq.). The vial was sealed and shaken for 4 days at 60°C. The reaction mixture was transferred to a separatory funnel with distilled water and CH_2Cl_2 . The aqueous phase was made basic with sodium hydrogen carbonate and extracted with dichloromethane (3 times). The organic layers were collected and dried
30 with sodium sulfate and concentrated, this resulted in a yellow oil. The product was purified by flash chromatography (3% MeOH in CH_2Cl_2). The resulting product was dissolved in dichloromethane (1 ml) and HCl (1 eq. 2 M HCl in ether) was added under stirring. The salt precipitated by addition of the dichloromethane solution into

heptane. Concentration on the rotary evaporator returned the product as white crystals. Yield 101.2 mg (63%), UV/MS 94/96 (M^+ 415), R_f 0.25 (3% MeOH/ CH_2Cl_2).

1H -NMR (400 MHz, $CDCl_3$) δ 12.05 (brs, 1H), 7.36 (d, 2H), 7.18 (d, 2H), 7.04 (d, 2H), 6.82 (d, 2H), 4.88(m, 1H), 4.60 (s, 2H), 3.79 (s, 3H), 3.55 (d, 2H), 3.36 (d, 3H), 2.80 (brq, 2H), 2.65 (brq, 2H), 1.76 (brd, 2H), 1.39 (d, 6H). ^{13}C -NMR 173.0; 159.0; 137.0; 136.0; 129.7; 129.3; 127.4; 126.4; 114.5; 57.9; 55.5; 49.2; 48.2; 46.2; 40.5; 25.8; 16.9.

Example 54 - 2-(Phenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH14b)

Procedure as for 50ELH27. Purification was done by HPLC. The hydrochloride salt was made by dissolving the free-base in dichloromethane (1 ml) and HCl (1 eq. 2 M HCl in ether) was added under stirring. The salt was precipitated by addition of the dichloromethane solution into heptane followed by concentration.

Reaction-step 1: 4-(4-Trifluoromethylbenzylamino)-1-methylpiperidin (50ELH2).

Starting materials: 1-Methyl-4-piperidone (1.13 g, 10.0 mmol, 1.0 eq.), 4-trifluoromethylbenzylamine (1.75 g, 1.0 eq.).

Product: UV/MS 80/92 (M^+ 273).

Reaction-step 2: 2-(Phenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH14b).

Starting materials: 50ELH2 (0.12 g, 0.44 mmol, 1.0 eq.), phenylacetylchloride (0.068 g, 1.0 eq.).

Product: UV/MS 100/97 (M^+ 390), r_t (A, MS) 3.797, R_f 0.3

(5%MeOH/ CH_2Cl_2). 1H -NMR (400 MHz, $CDCl_3$, rotamers 54/46) δ 7.52 (d, 2H), 7.42 (d, 2H), 7.12-7.30 (m, 4H), 4.63 and 3.74 (2m, 1H), 4.38 (brs, 2H), 3.80 and 3.50 (2s, 3H), 3.31 and 2.78 (2d, 2H), 2.33 and 2.18 (2s, 2H), 2.24 and 1.65-1.90 (t and m, 4H), 1.60 and 1.22 (2d, 2H), 1. ^{13}C -NMR 172.3; 171.8; 143.9; 135.1; 134.8; 129.1; 129.0; 128.9; 128.7; 127.4; 127.3; 127.2; 126.3; 126.1; 126.0; 56.0; 55.2; 54.9; 50.9; 46.8; 45.2; 44.9; 42.2; 41.7; 30.6; 28.4.

Example 55 - 2-(4-Fluorophenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH14c)

Procedure as 50ELH14B.

Reaction-step 2: 2-(4-Fluorophenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH14c).

Starting materials: 50ELH2 (0.12 g, 0.44 mmol, 1.0 eq.), 4-fluorophenylacetylchloride (0.076 g, 1.0 eq.).

5 *Product:* Yield 69.7 mg (36%), UV/MS 100/98 (M^+ 409), r_f (A, MS) 3.839, R_f 0.3 (5%MeOH/CH₂Cl₂). ¹H-NMR (400 MHz, DMSO, rotamers 65/35) δ 10.80 and 10.60 (2s, 1H), 7.71 and 7.62 (2d, 2H), 7.47 and 7.38 (2d, 2H), 7.00-7.36 (t and m, 4H), 4.70 and 4.50 (2s, 2H), 4.30 (m, 1H), 3.93 and 3.56 (2s, 2H), 3.34 (s, 2H), 3.00 (brq, 2H), 2.64 (s, 3H), 2.08 (m, 2H), 1.68 and 1.58 (2d, 2H). ¹³C-NMR 176.8; 176.4; 10
167.6; 165.3; 150.0; 149.0; 136.6; 132.5; 131.0; 130.5; 120.6; 120.5; 120.5; 120.4; 58.1; 58.0; 57.0; 54.5; 52.0; 49.3; 47.6; 45.0; 32.4; 31.4.

Example 56 - 2-(4-Methoxyphenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH14d)

Procedure as 50ELH14B.

15 Reaction-step 2: 2-(4-Methoxyphenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH14d).

Starting materials: 50ELH2 (0.15 g, 0.55 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.1 g, 1.0 eq.).

Product: Yield 57.5 mg (29%), UV/MS 99/100 (M^+ 421), r_f (B, MS) 6.30, R_f 0.25 (3%MeOH/CH₂Cl₂). ¹H-NMR (400 MHz, CDCl₃) δ 12.4 (brs, 1H), 7.55 (d, 2H), 7.28 (d, 2H), 6.96 (d, 2H), 4.84 (brt, 1H), 4.59 (s, 2H), 3.72 (s, 3H), 3.46 (s, 2H), 3.38 (d, 2H), 2.78 (q, 2H), 2.64 (s, 3H), 2.38 (q, 2H), 1.70 (d, 2H). ¹³C-NMR 173.0; 159.0; 142.3; 130.0; 129.8; 126.3; 126.2; 114.7; 114.5; 55.5; 54.4; 48.7; 46.5; 43.6; 40.6; 26.3.

25 **Example 57 - 2-(4-Trifluoromethylphenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH14a)**

Procedure as 50ELH14B.

Reaction-step 2: 2-(4-Trifluoromethylphenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH14a).

30 *Starting materials:* 50ELH2 (0.12 g, 0.44 mmol, 1.0 eq.), 4-trifluoromethylphenylacetylchloride (0.1 g, 1.0 eq.).

Product: Yield 92.6 mg (42%), UV/MS 89/93 (M^+ 458), r_f (A, MS) 4.211, R_f 0.3 (5%MeOH/CH₂Cl₂). ¹H-NMR (400 MHz, CDCl₃) δ 12.7 (brs, 1H), 7.56 (d, 2H),

7.48 (d, 2H), 7.17 (d, 2H), 4.86 (m, 1H), 4.63 (s, 2H), 3.58 (s, 3H), 3.40 (d, 2H), 2.75 (q, 2H), 2.65 (d, 3H), 2.46 (dq, 2H), 1.73 (brs, 2H). ¹³C-NMR 171.8; 141.9; 138.4; 129.4; 127.9; 126.3; 126.3; 126.2; 125.9; 125.8; 54.4; 48.8; 46.6; 43.6; 40.9; 26.2.

Example 58 - 2-(4-Fluorophenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH6) Procedure as 50ELH14B.

Reaction-step 1: 4-(4-Fluorobenzylamino)-1-methylpiperidine (50ELH4).

Starting materials: 1-Methyl-4-piperidone (1.13 g, 10.0 mmol, 1.0 eq.), 4-fluorobenzylamine (1.25 g, 1.0 eq.).

Product: Yield 2.154 g (97%), UV/MS 79/89 (M⁺ 223).

10 Reaction-step 2: 2-(4-Fluorophenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH14a).

Starting materials: 50ELH4 (0.12 g, 0.54 mmol, 1.0 eq.), 4-fluorophenylacetylchloride (0.096 g, 1.0 eq.).

Product: Yield 57 mg (29%), UV/MS 100/100 (M⁺ 359), r_f (A, MS) 3.763, R_f 0.25 (3% MeOH/CH₂Cl₂). ¹H-NMR (400 MHz, CDCl₃) δ 12.6 (brs, 1H), 7.2 (dd, 2H), 7.06 (m, 4H), 6.98 (t, 2H), 4.88 (tt, 1H), 4.58 (s, 4H), 3.45 (d, 2H), 2.81 (q, 2H), 2.72 (d, 3H), 2.48 (brq, 2H), 1.78 (brs, 2H). ¹³C-NMR 172.5; 163.4; 160.8; 133.4; 130.6; 130.2; 127.5; 127.4; 116.3; 116.1; 115.9; 115.7; 54.5; 48.8; 46.2; 43.6; 40.3; 26.3.

20 Example 59 - 2-(4-Methoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH8).

Procedure as 50ELH14B

Reaction-step 2:

Starting materials: 50ELH4 (0.12 g, 0.54 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.1 g, 1.0 eq.).

Product: Yield 54 g (26%), UV/MS 100/100 (M⁺ 371), r_f (A, MS) 3.257, R_f 0.25 (3% MeOH/CH₂Cl₂). ¹H-NMR (400 MHz, CDCl₃) δ 12.2 (brs, 1H), 7.12 (m, 2H), 6.97 (m, 4H), 6.75 (d, 2H), 4.80 (brt, 1H), 4.49 (s, 2H), 3.71 (s, 3H), 3.47 (s, 2H), 3.37 (d, 2H), 2.8 (q, 2H), 2.64 (s, 3H), 2.35 (q, 2H), 1.69 (d, 2H). ¹³C-NMR 173.0; 163.5; 161.1; 158.9; 133.7; 133.6; 129.8; 127.6; 127.5; 126.5; 116.2; 116.0; 114.6; 114.5; 55.5; 54.4; 48.8; 46.2; 43.6; 40.5; 26.4.

Example 60 – 2-(Phenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH10)

Procedure as 50ELH14B.

Reaction-step 2: 2-(Phenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl)

5 acetamide (50ELH10).

Starting materials: 50ELH4 (0.13 g, 0.59 mmol, 1.0 eq.), phenylacetylchloride (0.091 g, 1.0 eq.).

Product: UV/MS 100/94 (M^+ 341), r_t (A, MS) 3.127, R_f 0.25

(3%MeOH/CH₂Cl₂). ¹H-NMR (400 MHz, DMSO, rotamers 54/56) δ 12.38 (brs, 1H),
 10 7.35-7.00 (m, 9H), 4.55 and 4.40 (2s, 2H), 4.50 and 4.25 (brt, 1H), 3.91 and 3.56 (2s, 2H), 3.30 (Hidden under water signal)(2H), 2.98 (d, 2H), 2.64 (s, 3H), 2.09 (brt, 2H), 1.66 and 1.45 (2brd, 2H). ¹³C-NMR 171.9; 171.6; 162.8; 160.4; 136.5; 136.2; 135.4; 129.9; 129.7; 129.5; 129.2; 129.0; 128.9; 128.7; 127.2; 127.1; 116.2; 116.0; 115.6; 53.2; 52.5; 49.8; 46.9; 44.0; 42.8; 40.9; 40.6; 40.4; 40.2; 40.0; 39.8; 39.6; 27.7; 26.6.

15 **Example 61 – 2-(4-Trifluoromethylphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH12²)**

Procedure as 50ELH14B.

Reaction step 0: 4-Trifluoromethylphenylacetyl chloride (50ELH12¹)

4-Trifluorophenylacetic acid (1.0 g) and thionyl chloride (15 ml) were
 20 refluxed for 1 h. The excess thionyl chloride was evaporated off. NMR showed complete conversion.

Reaction-step 2: 2-(4-Trifluoromethylphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH12²).

Starting materials: 50ELH4 (0.12 g, 0.55 mmol, 1.0 eq.), 4-
 25 trifluoromethylphenylacetylchloride (50ELH12¹)(0.11 g, 0.5 mmol, 1.0 eq.).

Product: Yield 47.1 mg (24%), UV/MS 96/96 (M^+ 409), r_t (A, MS) 4.566, R_f 0.25 (3%MeOH/CH₂Cl₂). ¹H-NMR (400 MHz, CDCl₃) δ 7.52 (d, 2H), 7.22 (d, 2H), 7.17 (dd, 2H), 7.04 (t, 2H), 4.86 (brt, 1H), 4.58 (s, 2H), 3.64 (s, 2H), 3.45 (brd, 2H), 2.84 (brq, 2H), 2.71 (d, 3H), 2.45 (brq, 2H), 1.77 (brd, 2H). ¹³C-NMR 171.8; 163.6;
 30 161.2; 138.7; 133.3; 129.8; 129.5; 127.5; 127.4; 125.8; 125.7; 116.4; 116.2; 54.4; 48.9; 46.3; 43.6; 40.8; 26.3.

Example 62 – 4-(4-Methoxybenzylamino)-1-methylpiperidine (50ELH18).

Procedure as 50ELH27.

Starting materials: 1-Methyl-4-piperidone (1.13 g, 10.0 mmol, 1.0 eq.), 4-methoxybenzylamine (1.37 g, 1.0 eq.).

Product: UV/MS 95/95 (M^+ 235), r_f (A, MS) 3.509. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 7.3-6.8 (m, 4H), 3.77 (s, 3H), 3.73 (s, 2H), 2.86 (m, 2H), 2.55 (m, 1H), 2.30 (s, 3H), 2.1 (t, 2H), 1.96 (dd, 2H), 1.50 (m, 2H).

Example 63 – 2-(4-Trifluoromethylphenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH20A)

Procedure as 50ELH14B.

Reaction-step 1: Methyl 4-(N-[1-methylpiperidine-4-yl] aminomethyl) benzoate (50ELH19).

Starting materials: 1-Methyl-4-piperidone (1.13 g, 10.0 mmol, 1.0 eq.), methyl 4-(aminomethyl) benzoate hydrochloride (2.0 g, 1.0 eq.).

Product: UV/MS 81/88 (M^+ 263), r_f (A, MS) 3.060. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 8.00 (d, 2H), 7.20 (d, 2H), 3.90 (s, 3H), 3.85 (s, 2H), 2.96 (dt, 2H), 2.7 (brs, 1H), 2.62 (m, 1H), 2.40 (s, 3H), 2.28 (t, 2H), 1.96 (m, 2H), 1.56 (m, 2H).

Reaction-step 2: 2-(4-Trifluoromethylphenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH20A).

Starting materials: 50ELH19 (0.20 g, 0.76 mmol, 1.0 eq.), 50ELH12¹ (0.169 g, 1.0 eq.).

Product: Yield 108.9 mg (32%), UV/MS 100/100 (M^+ 448), r_f (A, MS) 3.327, R_f 0.3 (5% MeOH/ CH_2Cl_2). $^1\text{H-NMR}$ (400 MHz, DMSO, rotamers 56/44) δ 10.7 and 10.4 (2brs, 1H), 7.96-7.28 (m, 8H), 4.70 and 4.51 (2s, 2H), 4.30 (brt, 1H), 4.06 and 3.69 (2s, 2H), 3.83 and 3.81 (2s, 3H), 3.00 (m, 2H), 2.63 (m, 3H), 2.05 (brt, $J=12$ Hz, 2H), 1.69 (brt, $J=12$ Hz, 2H). $^{13}\text{C-NMR}$ (CDCl_3) 171.9; 166.7; 142.9; 138.5; 130.7; 130.1; 129.7; 126.2; 125.9; 55.2; 52.5; 49.2; 47.4; 41.2; 32.1; 26.6; 22.9; 14.3.

Example 64 – 2-Phenyl-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH20B)

Procedure as 50ELH14B

Reaction-step 2: 2-Phenyl-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH20B)

Starting materials: 50ELH19 (0.2 g, 0.76 mmol, 1.0 eq.), phenylacetylchloride (0.117 g, 1.0 eq.).

Product: Yield 82.5 g (29%), UV/MS 100/100 (M^+ 381), r_t (A, MS) 2.652, R_f 0.25 (3%MeOH/CH₂Cl₂). ¹H-NMR (400 MHz, CDCl₃) δ 12.2 (brs, 1H), 8.00 (d, $J=7.4$, 2H), 7.4-7.2 (m, 4H), 7.08 (d, $J=7.4$, 2H), 4.89 (brt, 1H), 4.62 (s, 2H), 3.90 (s, 3H), 3.56 (s, 2H), 3.42 (d, $J=11.0$, 2H), 2.84 (q, $J=11.0$, 2H), 2.68 (d, $J=3.6$, 3H), 2.40 (q, $J=11.0$, 2H), 1.77 (brd, $J=11.0$, 2H). ¹³C-NMR 173.0; 168.0; 143.3; 136.7; 130.6; 129.0; 127.4; 125.9; 54.5; 52.4; 48.8; 43.6; 41.4; 26.3.

Example 65 – 2-(4-Chlorophenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH20C).

Procedure as 50ELH14B.

10 Reaction-step 2: 2-(4-Chlorophenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH20C).

Starting materials: 50ELH19 (0.2 g, 0.76 mmol, 1.0 eq.), 4-chlorophenylacetylchloride (0.131 g, 1.0 eq.).

Product: Yield 79.2 g (26%), UV/MS 100/96 (M^+ 399), r_t (A, MS) 2.333. ¹H-NMR (400 MHz, DMSO, rotamers 62/38) δ 10.8 and 10.60 (2brs, 1H), 7.95 and 7.85 (2d, $J=8.6$, 2H), 7.4 and 7.28 (2d, 2H), 7.35 and 7.14 (2m, 4H), 4.67 and 4.50 (2s, 2H), 4.29 (m, 1H), 3.93 and 3.84 (2s, 2H), 3.81 (s, 3H), 3.21 (d, $J=11.9$, 2H), 3.00 (d, $J=11.9$, 2H), 2.63 (s, 3H), 2.06 (m, 2H), 1.68 and 1.56 (d, $J=11.9$, 2H). ¹³C-NMR (CDCl₃) 172.6; 166.7; 163.4; 161.0; 143.0; 130.7; 130.6; 130.5; 126.0; 115.9; 115.7; ; 54.7; 52.4; 48.9; 46.9; 44.0; 40.4; 26.4.

Example 66 – 2-(4-Methoxyphenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH20D).

Procedure as 50ELH14B.

25 Reaction-step 2: 2-(4-Methoxyphenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH20D).

Starting materials: 50ELH19 (0.2 g, 0.76 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.140 g, 1.0 eq.).

Product: Yield 108.6 g (26%), UV/MS 100/99 (M^+ 410), r_t (A, MS) 2.280. ¹H-NMR (400 MHz, CDCl₃) δ 12.38 (brs, 1H), 8.00 (d, $J=7.2$, 2H), 7.28 (d, $J=7.2$, 2H), 7.00 (d, $J=7.2$, 2H), 6.79 (d, $J=7.2$, 2H), 4.88 (brt, 1H), 4.61 (s, 2H), 3.90 (s, 3H), 3.75 (s, 3H), 3.42 (brd, $J=10.7$, 2H), 2.84 (q, $J=10.7$, 2H), 2.68 (d, $J=3.6$, 3H), 2.40 (brq, $J=10.7$, 2H), 1.75 (d, $J=10.7$, 2H). ¹³C-NMR 173.0; 166.8; 159.0; 143.3;

130.5; 129.9; 129.8; 126.3; 125.9; 114.5; 55.5; 54.7; 52.4; 48.7; 46.7; 43.6; 40.6;
32.1; 26.3; 22.9; 14.3.

Example 67 – 2-(4-TMethylphenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH23)

5 Procedure as 50ELH14B.

Reaction-step 2: 1-Phenyl-N-[2-(4-methylphenyl)ethyl]-N-(1-methylpiperidin-4-yl) amide (50ELH23).

Starting materials: 4-(2-Phenylethyl)amino-1-methylpiperidine (0.20 g, 0.86 mmol, 1.0 eq.), benzoylchloride (0.158 g, 1.0 eq.).

10 *Product:* Yield 159 mg (50%), UV/MS 100/100 (M^+ 337), r_t (A, MS) 3.289, R_f 0.55 (10% MeOH/ CH_2Cl_2). 1H -NMR (400 MHz, DMSO (80°C)) δ 10.9 (brs, 1H), 7.44 (s, 2H), 7.34 (d, $J=3.0$ Hz, 2H), 7.04 (d, $J=7.0$ Hz, 2H), 6.95 (brs, 2H), 4.00 (brs, 1H), 3.40 (d, $J=4.2$ Hz, 2H), 3.35 (d, $J=4.2$ Hz, 2H), 2.95 (brs, 2H), 2.77 (t, $J=3.2$ Hz, 2H), 2.40 (q, $J=6.4$ Hz, 2H), 2.24 (s, 3H) 1.83 (d, $J=6.4$ Hz, 2H). ^{13}C -NMR ($CDCl_3$)
15 171.6; 138.1; 136.3; 136.0; 129.8; 129.6; 129.1; 129.1; 126.7; 53.6; 52.4; 46.1; 42.9; 35.9; 27.3; 21.1.

Example 68 – 2-(4-Methoxyphenyl)-N-(3-phenyl-1-propyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH65)

Procedure as 50ELH14B.

20 Reaction-step 1: 4-(3-Phenylaminopropyl)piperidine (50ELH59)

Starting materials: 1-Methyl-4-piperidone (1.1 ml, 7.4 mmol, 1.0 eq.), 3-phenylpropylamine (1.35 g, 1.0 eq.).

Product: UV/MS 100/94 (M^+ 233), r_t (A, MS) 3.534. 1H -NMR (400 MHz, $CDCl_3$) δ 7.28-7.12 (m, 5H), 3.40 (brs, 1H), 2.84 (dt, $J=12.3$ and 3.5 Hz, 2H), 2.64 (q, $J=7.0$ Hz, 4H), 2.51 (m, 1H), 2.27 (s, 3H), 2.05 (brt, $J=12.3$ Hz, 2H), 1.82 (m, 2H),
25 1.44 (m, 2H).

Reaction-step 2: 2-(4-Methoxyphenyl)-N-(3-phenyl-1-propyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH65)

Starting materials: 50ELH59 (0.50 g, 2.2 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.398 g, 1.0 eq.).
30

Product: Yield 153 mg (43%), UV/MS 100/100 (M^+ 381), r_t (A, MS) 2.938. 1H -NMR (400 MHz, DMSO, rotamers 55/45) δ 11.0 and 10.90 (2brs, 1H), 7.30-7.10 (m, $J=7.9$ Hz, 6H), 6.97 (d, $J=7.9$ Hz, 1H), 4.22 and 4.06 (2dt, dH), 3.70 (s, 3H), 3.35

(t, $J=10.4$ Hz, 2H), 3.15 (m, 2H), 3.00 (q, $J=10.4$ Hz, 2H), 2.66 (d, 3H), 2.52 (q, $J=7.9$ Hz, 2H), 2.17 (brq, $J=12$ Hz, 2H) 1.73 (m, 2H), 1.70 and 1.52 (2d, $J=12$ Hz, 2H). ^{13}C -NMR (DMSO) 171.3; 171.0; 158.6; 142.2; 141.7; 130.0; 129.0; 128.0; 128.5; 128.2; 126.6; 114.5; 55.7; 55.7; 53.5; 53.3; 50.1; 44.5; 42.9; 41.9; 33.7; 33.1; 32.9; 31.4; 27.8; 26.8.

Example 69 – 2-(4-Methoxyphenyl)-N-[2-(4-methylphenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH68)

Procedure as 50ELH14B

Reaction-step 1: 4-[2-(4-Methylphenyl)ethylamino]-piperidin (50ELH58)

10 *Starting materials:* 1-Methyl-4-piperidone (1.1 ml, 7.4 mmol, 1.0 eq.), 2-(4-methylphenyl)ethylamine (1.0 g, 1.0 eq.).

Product: UV/MS 100/91 (M^+ 233), r_t (A, MS) 3.933. ^1H -NMR (400 MHz, CDCl_3) δ 7.4 (s, 5H), 3.27 (brs, 1H), 2.84 (d, $J=7.0$ Hz, 4H), 2.75 (m, 2H), 2.54 (m, 1H), 2.29 (2xs, 6H), 2.10 (brt, $J=12.3$ Hz, 2H), 1.86 (brd, 2H), 1.45 (m, 2H).

15 Reaction-step 2: 2-(4-Methoxyphenyl)-N-[2-(4-methylphenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH68)

Starting materials: 50ELH58 (0.30 g, 1.3 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.238 g, 1.0 eq.).

20 *Product:* Yield 125 mg (26%), UV/MS 100/99 (M^+ 381), r_t (A, MS) 3.156. ^1H -NMR (400 MHz, DMSO, rotamers 50/50) δ 11.0 and 10.90 (2brs, 1H), 7.25-7.04 (m, $J=8.7$ Hz, 6H), 6.87 and 6.84 (2d, $J=8.7$ Hz, 2H), 4.30 and 4.09 (2dt, $J=11.5$ Hz, dH), 3.73 and 3.58 (2s, 2H), 3.71 and 3.70 (2s, 3H), 3.35 (m, (Underneath waterpeak) 3H), 3.24 (m, 1H), 3.02 (m, $J=11.5$ Hz, 2H), 2.80-2.62 (m, 5H), 2.32 and 2.20 (2q, $J=11.5$ Hz, 2H), 2.26 and 2.24 (2s, 3H) 1.78 and 1.49 (2d, $J=11.5$ Hz, 2H). ^{13}C -NMR (DMSO) 171.5; 171.2; 158.6; 136.8; 136.2; 136.0; 135.8; 130.7; 130.5; 129.7; 129.6; 129.4; 129.2; 128.4; 128.3; 114.5; 55.8; 55.7; 53.3; 53.3; 52.2; 50.2; 46.8; 43.9; 42.9; 36.8; 35.2; 27.6; 26.8; 21.3.

Example 70 – 2-(4-Methoxyphenyl)-N-[2-(2-thiony)ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH71A)

30 Procedure as 50ELH14B

Reaction-step 1: 4-[2-(2-Thienyl)ethylamino]piperidin (50ELH67A)

Starting materials: 1-Methyl-4-piperidone (0.5 g, 4.4 mmol, 1.0 eq.), thiophene-2-ethylamine (0.563 g, 1.0 eq.).

Product: UV/MS 94/93 (M^+ 225).

Reaction-step 2: 2-(4-Methoxyphenyl)-N-[2-(2-thienylethyl)]-N-(1-methylpiperidin-4-yl) acetamide (50ELH71A)

Starting materials: 50ELH67A (0.243 g, 1.08 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.2 g, 1.0 eq.).

Product: Yield 80.7 mg (33%), UV/MS 100/100 (M^+ 373), r_t (A, MS) 2.613. $^1\text{H-NMR}$ (400 MHz, DMSO, rotamers 50/50) δ 10.8 and 10.6 (2brs, 1H), 7.36 and 7.31 (2d, $J=4.7$ Hz, 1H), 7.20 and 7.06 (2d, $J=8.3$ Hz, 2H), 7.00-6.92 (m, $J=4.7$ and 2.8 Hz, 2H), 6.87 and 6.40 (2d, $J=8.3$ Hz, 2H), 4.22 and 4.08 (2dt, $J=12.2$ Hz, 1H), 3.71 (s, 3H), 3.70 (s, 2H), 3.46-3.30 (m, 4H), 3.10-2.90 (m, 4H), 2.67 (m, 2H), 2.28 and 2.12 (2q, $J=12$ Hz, 2H), 1.80 and 1.50 (2d, $J=12$ Hz, 2H). $^{13}\text{C-NMR}$ (DMSO) 172.5; 158.9; 139.6; 130.0; 129.6; 126.8; 124.5; 114.5; 55.5; 54.7; 49.3; 45.8; 43.8; 41.3; 31.9; 29.9

Example 71 – 2-(4-Methoxyphenyl)-N-[2-(4-nitrophenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH71C)

Procedure as 50ELH14B

Reaction-step 1: 4-[2-(4-nitrophenyl) ethylamino]-piperidin (50ELH67C)

Starting materials: 1-Methyl-4-piperidone (0.5 g, 4.4 mmol, 1.0 eq.), 4-nitrophenyl-2-ethylamine (0.897 g, 1.0 eq.).

Product: UV/MS 96/89 (M^+ 264), r_t (A, MS) 3.264.

Reaction-step 2: 2-(4-Methoxyphenyl)-N-[2-(4-nitrophenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH71A)

Starting materials: 50ELH67C (0.285 g, 1.08 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.2 g, 1.0 eq.).

Product: Yield 130.9 mg (30%), UV/MS 100/100 (M^+ 412), r_t (A, MS) 2.219.

$^1\text{H-NMR}$ (400 MHz, DMSO, rotamers 50/50) δ 10.8 and 10.6 (2brs, 1H), 8.17 and 8.12 (2d, $J=8.6$ Hz, 2H), 7.58 and 7.48 (2d, $J=8.6$ Hz, 2H), 7.2 and 7.1 (2d, $J=8.6$ Hz, 2H), 6.87 and 6.40 (2d, $J=8.6$ Hz, 2H), 4.25 and 4.10 (2dt, $J=12$ Hz, 1H), 3.72 (s, 3H), 3.70 (s, 2H), 3.48-3.30 (m, 4H), 3.10-2.84 (m, 4H), 2.69 and 2.67 (2d, $J=4.7$ Hz, 3H), 2.34 and 2.15 (2q, $J=13.2$ Hz, 2H), 1.79 and 1.47 (2d, $J=13.2$ Hz, 2H).

Example 72 – 2-(4-Methoxyphenyl)-N-(2-thienylmethyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH73A)

Procedure as 50ELH14B.

Reaction-step 1: 4-[(2-Thienylmethyl)amino]-1-methylpiperidine
(50ELH66A)

Starting materials: 1-Methyl-4-piperidone (0.5 g, 4.4 mmol, 1.0 eq.), 2-thienylmethylaniline (0.52 g, 1.0 eq.).

5 *Product:* UV/MS 77/86 (M^+ 211), r_t (A, MS) 2.739.

Reaction-step 2: 2-(4-Methoxyphenyl)-N-(2-thienylmethyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH73A)

Starting materials: 50ELH66A (0.228 g, 1.08 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.2 g, 1.0 eq.).

10 *Product:* Yield 178.4 mg (50%), UV/MS 100/98 (M^+ 359), r_t (A, MS) 3.117.

$^1\text{H-NMR}$ (400 MHz, DMSO) δ 10.9 and 10.6 (2brs, 1H), 7.47 and 7.32 (2d, $J=4.5$ Hz, 1H), 7.20 and 7.03 (2d, $J=8.4$ Hz, 2H), 7.03 and 6.98 (2m, 1H), 6.87 (m, 3H), 4.70 and 4.57 (2s, 2H), 4.42 and 4.16 (2t, $J=11.9$ Hz, 1H), 3.77 and 3.60 (2s, 2H), 3.51 (s, 3H), 3.15 (m, 2H), 2.98 (m, $J=11.9$ Hz, 2H), 2.65 (2d, $J=4.5$ Hz, 3H), 2.25 and 2.17 (2q, $J=11.9$ Hz, 2H), 1.69 and 1.44 (2d, $J=11.9$ Hz, 2H). $^{13}\text{C-NMR}$ (DMSO) 171.4; 158.6; 143.2; 130.7; 128.1; 126.6; 126.3; 125.9; 114.5; 55.7; 53.3; 52.6; 50.0; 42.8; 27.7; 26.8.

Example 73 – 2-(4-Methoxyphenyl)-N-(furfuryl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH73B).

20 *Procedure as* 50ELH14B.

Reaction-step 1: 4-(Furfurylamino)-1-methylpiperidin (50ELH66B)

Starting materials: 1-Methyl-4-piperidone (0.5 g, 4.4 mmol, 1.0 eq.), Furfurylamine (0.43 g, 1.0 eq.).

Product: UV/MS 77/92 (M^+ 195), r_t (A, MS) 2.812.).

25 Reaction-step 2: 2-(4-Methoxyphenyl)-N-(furfuryl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH73B).

Starting materials: 50ELH66B (0.21 g, 1.08 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.2 g, 1.0 eq.).

Product: Yield 134 mg (36%), UV/MS 100/99 (M^+ 343), r_t (A, MS) 2.401.

30 $^1\text{H-NMR}$ (400 MHz, DMSO, rotamers 57/43) δ 10.95 and 10.75 (2brs, 1H), 7.63 and 7.48 (s, 1H), 7.18 and 7.06 (2d, $J=7.7$ Hz, 2H), 6.85 (t, $J=7.7$ Hz, 2H), 6.44 and 6.33 (2d, $J=7.7$ Hz, 1H), 6.37 and 6.11 (2s, 1H) 4.5 and 4.34 (2s, 2H), 4.42 and 4.18 (2dt, $J=11$ and 2 Hz, 1H), 3.75 and 3.65 (2s, 2H) 3.70 (s, 3H), 3.33 (hidden, 2H), 3.0 (q,

2H), 2.64 (d, $J=4.7$ Hz, 3H), 2.15 (dq, $J=11$ and 2 Hz, 2H), 1.65 and 1.50 (2d, $J=11$ Hz, 2H).

Example 74 – 2-(2-thienylmethyl)-N-(4-methylphenylmethyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH82)

5 Procedure as 50ELH14B

Reaction-step 2: 2-(2-thienylmethyl)-N-(4-methylphenylmethyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH82)

Starting materials: 50ELH25 (0.30 g, 1.38 mmol, 1.0 eq.), thiophene-2-acetylchlorid (0.22 g, 1.0 eq.).

10 *Product:* Yield 235 mg (62%), UV/MS 97/93 (M^+ 343), r_t (A, MS) 2.795. 1H -NMR (400 MHz, DMSO, rotamers 54/46) δ 10.8 and 10.60 (2brs, 1H), 7.4 and 7.35 (2d, 1H), 7.2-6.76 (m, 6H), 4.55 and 4.4 (2s, 2H), 4.49 and 4.26 (2dt, $J=11$ and 2 Hz, 2H), 4.15 and 3.79 (2s, 2H), 3.32 (d, $J=11$ Hz, 2H), 2.99 (q, 2H), 2.63 (s, 3H), 2.27 and 2.23 (2s, 3H), 2.09 (q, $J=11$ Hz, 2H), 1.66 and 1.55 (2d, $J=11$ Hz, 2H).

15 **Example 75 – 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-cyclopentylpiperidin-4-yl) acetamide (42ELH75)**

Procedure as for 42ELH80, except that the reaction was run at 60°C for 3 days.

Starting materials: 50ELH87 (0.25 g, 0.71 mmol, 1.0 eq.),

20 Cyclopentylbromide (0.288 g, 3.0 eq.).

Product: Yield 91.2 mg (34%), UV/MS 88/93 (M^+ 421), r_t (A, MS) 4.450.

Example 76 – 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-(3-(1,3-dihydro-2H-benzimidazol-2-one-1-yl)propyl)piperidine-4-yl) acetamide (50ELH89).

25 50ELH87 (0.05 g, 0.14 mmol, 1 eq.) was transferred to a 4 ml vial and dissolved in 1 ml of acetonitrile. Then, 1-(3-chloropropyl)-1,3-dihydro-2H-benzimidazol-2-one (0.032 g, 1.1 eq.), sodium carbonate (0.022 g, 1.1 eq.) and KI (one crystal) were added and the vial was sealed and shaken for 20 h at 82°C. The mixture was extracted with distilled water (pH 10, sodium carbonate) and
30 dichloromethane (3 times) the organic layers were dried with sodium sulfate and concentrated. The title compound was purified by HPLC and evaporated to dryness, forming a trifluoroacetic acid salt. Yield 8.8 mg (12%). UV/MS 100/100 (M^+ 527), r_t (A, MS) 2.851.

Example 77 – 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(2-methylthiazol-4-ylmethyl) piperidin-4-yl] acetamide (63ELH1A).

50ELH87 (0.3 g, 0.852 mmol, 1.0 eq) and 4-(chloromethyl)-2-methylthiazole hydrochloride (0.235 g, 1.5 eq) were added to a 7 ml vial and dissolved in acetonitrile (3ml). Potassium carbonate (141.3 g, 1.2 eq) and a crystal of potassium iodide were added and the vial was sealed and shaken for 20 h at 82°C. The reaction mixture was extracted with distilled water (made basic by potassium carbonate, pH 10) and dichloromethane. The crude product was dried with sodium sulfate and concentrated. After purification by HPLC the product was converted into the hydrochloride salt by dissolving the free base in 1 ml dichloromethane and adding 1 eq. HCl in ether (2M). This mixture was added drop-wise to an excess of heptane where the product precipitated. The solvent was removed by evaporation leaving a white powder as the product. yield 83.8 mg (21%), UV/MS 100/90 (M^+ 463), r_f (B, MS) 11.82.

Example 78 – 2-(4-Methoxyphenyl)-N-(2-4-(fluorophenyl) ethyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH93A)

Procedure as 50ELH14B.

Reaction-step 1: 4-[2-4-(Fluorophenyl)ethylamino]-1-methylpiperidine (50ELH92A)

Starting materials: 1-Methyl-4-piperidone (0.3 g, 2.65 mmol, 1.0 eq.), 4-(fluorophenyl)ethylamine (0.369 g, 1.0 eq.).

Product: UV/MS 60/92 (M^+ 237), r_f (A,MS) 3.422.

Reaction-step 2: 2-(4-Methoxyphenyl)-N-(2-4-(fluorophenyl)ethyl)-N-(1-methylpiperidin-4-yl) acetamide (50ELH93A)

Starting materials: 50ELH92A (0.625 g, 2.65 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.488 g, app. 1.0 eq.).

Product: Yield 181 mg (18%), UV/MS 87/97 (M^+ 385), r_f (A, MS) 2.783. R_f 0.8 (10% MeOH/ CH_2Cl_2). 1H -NMR (400 MHz, DMSO, rotamers 50/50) δ 10.9 (brs, 1H), 7.56-6.8 (m, 8H), 4.26 and 4.02 (2brt, 2H), 3.70 and 3.95 (2s, 3H), 3.59 and 3.57 (2s, 2H), 3.4-3.15 (m, 5H), 2.96-2.66 (m, 5H), 2.62 and 2.56 (2s, 3H), 2.29 and 2.10 (2q, 2H), 1.73 and 1.41 (2d, 2H). ^{13}C -NMR (DMSO) 172.5; 171.4; 171.3; 162.9; 162.7; 160.5; 160.3; 158.9; 158.6; 136.1; 136.1; 135.3; 131.4; 131.3; 131.1; 131.0; 131.0; 130.6; 130.5; 128.4; 128.4; 126.9; 115.9; 115.8; 115.7; 115.6; 114.5; 55.7; 53.7; 53.5; 52.7; 52.3; 50.7; 46.7; 43.8; 43.2; 43.0; 36.3; 34.7; 27.9; 26.9.

Example 79 – 2-(4-Methoxyphenyl)-N-[2-(2,5-dimethoxyphenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH93C)

Procedure as 50ELH14B. A small amount was purified by HPLC and evaporated to dryness, forming the trifluoroacetic acid salt.

5 Reaction-step 1: 4-[2-(2,5-dimethoxyphenyl)ethylamino]-1-methylpiperidine (50ELH92A)

Starting materials: Methyl-4-piperidone (0.3 g, 2.65 mmol, 1.0 eq.), 2,5-(dimethoxyphenyl)ethylamine (0.481 g, 1.0 eq.).

Product: UV/MS 81/90 (M^+ 279), r_t (A,MS) 2.868.

10 Reaction-step 2: 2-(4-Methoxyphenyl)-N-[2-(2,5-dimethoxyphenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH93C)

Starting materials: 50ELH93C (0.737 g, 2.65 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.488 g, app. 1.0 eq.).

15 *Product:* UV/MS 82/100 (M^+ 427), r_t (B, MS) 8.44. R_f 0.8 (10% MeOH/CH₂Cl₂).

Example 80 – 2-(4-Methoxyphenyl)-N-[2-(2,4-dichlorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH93D)

Procedure as 50ELH14B, but purified by HPLC and evaporated to dryness forming the trifluoroacetic acid salt.

20 Reaction-step 1: 4-[2-(2,4-Dichlorophenyl)ethylamino]-1-methylpiperidine (50ELH92D)

Starting materials: 1-Methyl-4-piperidone (0.3 g, 2.65 mmol, 1.0 eq.), 2,5-(dichlorophenyl) ethylamine (0.50 g, 1.0 eq.).

Product: UV/MS 82/92 (M^+ 287), r_t (A,MS) 4.875.

25 Reaction-step 2: 2-(4-Methoxyphenyl)-N-[2-(2,4-dichlorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH93D)

Starting materials: 50ELH93D (0.76 g, 2.65 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.488 g, app. 1.0 eq.).

30 *Product:* UV/MS 100/96 (M^+ 435), r_t (A, MS) 4.415. R_f 0.8 (10% MeOH/CH₂Cl₂).

Example 81 – 2-(4-Methoxyphenyl)-N-[2-(3-chlorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH93E)

Procedure as 50ELH14B, but purified on HPLC and evaporated to dryness forming the trifluoroacetic acid salt.

Reaction-step 1: 4-[(3-Chlorophenyl)ethyl]amino]-1-methylpiperidine
(50ELH92E)

Starting materials: 1-Methyl-4-piperidone (0.3 g, 2.65 mmol, 1.0 eq.), 3-(chlorophenyl) ethylamine (0.413 g, 1.0 eq.).

5 *Product:* UV/MS 86/88 (M^+ 253), r_t (A, MS) 3.175.

Reaction-step 2: 2-(4-Methoxyphenyl)-N-[2-(3-chlorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH93E)

Starting materials: 50ELH93E (0.67 g, 2.65 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.488 g, app. 1.0 eq.).

10 *Product:* UV/MS 100/100 (M^+ 401), r_t (A, MS) 3.464. R_f 0.8 (10% MeOH/CH₂Cl₂).

Example 82 – 2-(4-Methoxyphenyl)-N-[2-(4-methoxyphenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH95B)

15 *Procedure as 50ELH14B. Purified by HPLC and evaporated to dryness forming the trifluoroacetic acid salt.*

Reaction-step 1: 4-[(4-Methoxyphenyl)ethyl]amino]-1-methylpiperidine
(50ELH94B)

Starting materials: 1-Methyl-4-piperidone (0.3 g, 2.65 mmol, 1.0 eq.), 4-methoxyphenylethylamine (0.40 g, 1.0 eq.).

20 *Product:* UV/MS 74/87 (M^+ 249), r_t (A, MS) 2.935.

Reaction-step 2: 2-(4-Methoxyphenyl)-N-[2-(4-methoxyphenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH95B)

Starting materials: 50ELH94B (0.657 g, 2.65 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.488 g, app. 1.0 eq.).

25 *Product:* UV/MS 100/100 (M^+ 397), r_t (A, MS) 2.389. R_f 0.8 (10% MeOH/CH₂Cl₂).

Example 83 – 2-(4-Methoxyphenyl)-N-[2-(3-fluorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH95D)

30 *Procedure as 50ELH14B. Purified on HPLC and evaporated to dryness, forming the trifluoroacetic acid salt.*

Reaction-step 1: 4-[2-((3-Fluorophenyl)ethyl)amino]-1-methylpiperidine
(50ELH94D)

Starting materials: 1-Methyl-4-piperidone (0.3 g, 2.65 mmol, 1.0 eq.), 3-fluorophenylethylamine (0.369 g, 1.0 eq.).

Product: UV/MS 74/89 (M^+ 237), r_f (A, MS) 2.946.

Reaction-step 2: 2-(4-methoxyphenyl)-N-[2-(3-fluorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide (50ELH95D)

Starting materials: 50ELH94D (0.625 g, 2.65 mmol, 1.0 eq.), 4-methoxyphenylacetylchloride (0.488 g, app. 1.0 eq.).

Product: UV/MS 100/95 (M^+ 385), r_f (A, MS) 2.946. R_f 0.8 (10% MeOH/CH₂Cl₂).

Example 84 – 2-(4-ethoxyphenyl)-N-[2-(4-fluorophenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide (63ELH20)

10 Reaction step 1: 4-Ethoxyphenylacetic acid chloride(63ELH19)

4-Ethoxyphenylacetic acid (0.5 g, 2.8 mmol) was transferred to a 7 ml vial and dissolved in thionylchloride (3 ml). The reaction mixture was shaken at 70°C for 2½ hours. Thionylchloride was evaporated off and the resulting product was used unpurified.

15 Reaction step 2: 2-(4-Ethoxyphenyl)-N-[2-(4-fluorophenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide (63ELH20)

63ELH17 (0.11 g, 0.47 mmol) was transferred to a 4 ml vial and dissolved in dichloromethane. 63ELH19 (0.084 mg, 1 eq.) was added and the vial was sealed and the reaction shaken for 20 h. The product was extracted in distilled water (made basic with potassium carbonate, pH 10) and dichloromethane. Dried with sodium sulfate and concentrated. Purified by HPLC. The extraction, drying and concentration was repeated and the product re-dissolved in dichloromethane (1 ml) and HCl (1 eq., 2 M in ether) was added. The mixture was added drop-wise to an excess of heptane whereupon the salt precipitated. Yield 33.4 mg (18%), UV/MS: 92/100 (M^+ 399), t_r 25 (B, MS) 10.38.

Example 85 – 2-(4-Ethoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide (63ELH21)

50ELH4 (0.11 g, 0.49 mmol, 1.0 eq.) was transferred to a 4 ml vial and dissolved in dichloromethane. 63ELH19 (0.089 mg, 1.0 eq.) was added and the vial 30 was sealed and the reaction shaken for 20 h. The product was extracted in distilled water (made basic with potassium carbonate, pH 10) and dichloromethane. Dried with sodium sulfate and concentrated. Purified by HPLC. The extraction, drying and concentration was repeated and the product dissolved in dichloromethane (1 ml) and HCl (1 eq., 2 M in ether) is added. This mixture was added drop-wise to an excess of

heptane whereupon the salt precipitated. Yield 31.1 mg (16%), UV/MS: 94/100 (M^+ 385), t_r (A, MS) 2.573.

Example 86 – *N*-((4-methylphenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-2-(3-hydroxy-4-methoxyphenyl)acetamide (57MBT12B)

5 *N*-((4-methylphenyl)methyl)-4-amino-1-methylpiperidine (50ELH25) (105 mg, 0.48 mmol) and 3-hydroxy-4-methoxyphenylacetic acid (88 mg, 0.48 mmol) were dissolved in DMF (10 ml). Diisopropylethylamine (DIEA, 250 μ L, 1.44 mmol) was added followed by bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBrOP, 336 mg, 0.72 mmol), and the mixture was stirred at r.t for 1 h. Water (50
10 mL) was added, and the reaction mixture was extracted with EtOAc (2 \times 50 mL). Drying by Na_2SO_4 and concentration yielded 514 mg crude material, which was purified by flash chromatography (0-30% MeOH in CH_2Cl_2). This gave 105 mg (57%) of the title compound as a white solid. R_f =0.20 (10% MeOH in CH_2Cl_2). HPLC-MS (method A) showed MH^+ =383. UV/MS(%)=100/92. 1H -NMR (400 MHz, CD_3OD , Rotamers 52:48): δ 7.18-6.58 (m, 7H), 4.53 (s, 2H), 4.31 and 3.97 (2m, 1H),
15 3.82 and 3.81 (2s, 3H), 3.80 and 3.55 (2s, 2H), 3.04 and 2.85 (2m, 2H), 2.41 and 2.32 (2s, 3H), 2.35 and 2.12 (2m, 2H), 2.29 and 2.27 (2s, 3H), 1.83 and 1.74 (2m, 2H), 1.72 and 1.33 (2m, 2H)

Example 87 – *N*-((4-methylphenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-2-(3,4-dihydroxyphenyl)acetamide (57MBT24B)

20 *N*-((4-methylphenyl)methyl)-*N*-(1-methylpiperidine-4-yl)-2-(3-hydroxy-4-methoxyphenyl)acetamide (57MBT12B) (52 mg, 0.136 mmol) was dissolved in CH_2Cl_2 (1 mL) and cooled to $-78^\circ C$. Boron tribromide (1M in CH_2Cl_2 , 204 μ L, 0.204 mmol) was added dropwise and the cooling bath was removed. After stirring for 2 h,
25 methanol (2 mL) was added and the mixture was evaporated. The resulting oil was purified by preparative HPLC to give 24 mg (48%) of the title compound as a white solid. HPLC-MS (method A) showed MH^+ =369. UV/MS(%)=100/97. 1H -NMR (400 MHz, CD_3OD , Rotamers 33:67): δ 7.19-6.47 (m, 7H), 4.54 and 4.53 (2s, 2H), 4.23 (m, 1H), 3.83 and 3.58 (2s, 2H), 3.46 and 3.40 (2br d, J =12 Hz, 2H), 3.02 and 2.95
30 (2br t, J =12 Hz, 2H), 2.79 (s, 3H), 2.33 and 2.28 (2s, 3H), 2.17 and 1.84 (2dq, J =4, 12 Hz, 2H), 1.87 and 1.48 (2br d, J =12 Hz, 2H)

Example 88 – *N*-((3-hydroxy-4-methylphenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-2-(4-methoxyphenyl)acetamide (57MBT54B)

N-((4-methoxyphenyl)methyl)-4-amino-1-methylpiperidine (1g, 4.27 mmol) was dissolved in 4% formic acid in methanol (60 mL). 10% Pd/C (1g) was added
5 under argon and the reaction mixture was heated to reflux for 24 h. The mixture was filtered through celite and the filtrate was acidified with conc. HCl to pH 1. Concentration yielded a yellow oil which was purified by flash chromatography (MeOH/CH₂Cl₂ 3:7 + 3.5% NH₄OH) to give 249 mg (51%) of 4-amino-1-methylpiperidine (**57-MBT36B**) as a white solid. *R*_f=0.13 (10% MeOH in CH₂Cl₂ +
10 3.5% NH₄OH). HPLC-MS (method B) showed MH⁺=115. UV/MS(%)=–/100.

4-Amino-1-methylpiperidine (**57MBT36B**) (26 mg, 0.231 mmol) was dissolved in methanol (1 mL) and 3-hydroxy-4-methylbenzaldehyde (32 mg, 0.231 mmol) and acetic acid (33 μL) were added. The mixture was cooled to 0 °C. NaBH₃CN (29 mg, 0.462 mmol) was added and the cooling bath was removed. After
15 3 h the reaction mixture was evaporated and flash chromatography (0-30% MeOH in CH₂Cl₂) gave 27 mg (50%) of *N*-((3-hydroxy-4-methylphenyl)methyl)-4-amino-1-methylpiperidine (**57MBT44C**) as a white solid. *R*_f=0.27 (10% MeOH in CH₂Cl₂ + 3.5% NH₄OH). HPLC-MS (method A) showed MH⁺=235. UV/MS(%)=99/99.

N-((3-hydroxy-4-methylphenyl)methyl)-4-amino-1-methylpiperidine
20 (**57MBT44C**) (27 mg, 0.115 mmol) was dissolved in CH₂Cl₂ (2 mL). 4-Methoxyphenylacetyl chloride (17 μL, 0.115 mmol) was added dropwise under argon. After 3 h, n-heptane (3 mL) was added and the mixture was evaporated. Flash chromatography (0-20% MeOH in CH₂Cl₂) gave 14 mg (32%) of the title compound as a white solid. *R*_f=0.32 (10% MeOH in CH₂Cl₂ + 3.5% NH₄OH). HPLC-MS
25 (method A) showed MH⁺=383. UV/MS(%)=99/96. ¹H-NMR (400 MHz, CD₃OD, Rotamers 63:37): δ 7.28-6.55 (m, 7H), 4.48 (s, 2H), 4.37 and 3.95 (2m, 1H), 3.78 and 3.77 (2s, 3H), 3.06 and 2.89 (2br d, *J*=12 Hz, 2H), 2.42 and 2.32 (2s, 3H), 2.40 and 2.12 (2m, 2H), 2.18 and 2.12 (2s, 3H), 1.86 and 1.83 (2m, 2H), 1.75 and 1.35 (2br d, *J*=12 Hz, 2H)

Example 89 – *N*-((4-methylphenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-2-(4-bromophenyl)acetamide hydrochloride(57MBT70-1D)

4-Bromophenylacetic acid (54 mg, 0.252 mmol) was dissolved in CH₂Cl₂ (2 mL), and *N*-((4-methylphenyl)methyl)-4-amino-1-methylpiperidine (292 mg/mL

stock solution in CH_2Cl_2 , 171 μL , 0.229 mmol) and polystyrene supported diisopropylethylamine (PS-DIEA with a loading of 3.57 mmol/g, 192 mg, 0.687 mmol) was added followed by bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBrOP, 160 mg/mL stock solution, 1 mL, 0.334 mmol). The
5 reaction mixture was shaken for 1 h at r.t. and filtered onto a prewashed (methanol) ion exchange column (0.88 mmol/g, 1 g). The column was washed with methanol (8*4 mL) and the remaining product was eluted off the column with 10% NH_4OH in methanol (2*4 mL) and evaporated. The resulting oil was filtered through silica (H=4 cm, D=1 cm) with methanol/ CH_2Cl_2 1:9 (20 mL), evaporated and subjected to a
10 second ion exchange column (0.88 mmol/g, 1 g). The column was washed with methanol (8*4 mL) and the remaining product was eluted off the column with 10% NH_4OH in methanol (2*4 mL) and evaporated on rotavap and oil pump. The product was dissolved in CH_2Cl_2 (0.5 mL) and HCl in diethylether (1.0 M, 0.1 mL, 0.1 mmol) was added. The solution was added to n-heptane (3 mL) and evaporation afforded 29
15 mg (25%) of the title compound as a white solid. $R_f=0.31$ (10% MeOH in CH_2Cl_2). HPLC-MS (method B) showed $\text{MH}^+=416$. UV/MS(%)=100/99.

Example 90 – N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-iodophenyl)acetamide hydrochloride(57MBT70-2D)

The title compound was prepared according to example MBT04. Yield: 33 mg
20 (26%). $R_f=0.31$ (10% MeOH in CH_2Cl_2). HPLC-MS (method B) showed $\text{MH}^+=463$. UV/MS(%)=100/98.

Example 91 – N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-(2-propyl)phenyl)acetamide hydrochloride(57MBT70-3D)

The title compound was prepared according to example MBT04. Yield: 36 mg
25 (34%). $R_f=0.31$ (10% MeOH in CH_2Cl_2). HPLC-MS (method B) showed $\text{MH}^+=379$. UV/MS(%)=100/97.

Example 92 – N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-trifluoromethoxyphenyl)acetamide hydrochloride(57MBT70-4D)

The title compound was prepared according to example MBT04. Yield: 35 mg
30 (30%). $R_f=0.27$ (10% MeOH in CH_2Cl_2). HPLC-MS (method B) showed $\text{MH}^+=421$. UV/MS(%)=100/99.

Example 93 – *N*-((4-methylphenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-2-(4-methylthiophenyl)acetamide hydrochloride(57MBT70-5D)

The title compound was prepared according to example MBT04. Yield: 35 mg (33%). $R_f=0.30$ (10% MeOH in CH_2Cl_2). HPLC-MS (method B) showed $\text{MH}^+=383$.
5 UV/MS(%)=100/99.

Example 94 – *N*-((4-methylphenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-2-(4-(*N,N*-dimethylamino)phenyl)acetamide hydrochloride(57MBT70-6D)

The title compound was prepared according to example MBT04.
Yield: 16 mg (15%). $R_f=0.25$ (10% MeOH in CH_2Cl_2). HPLC-MS (method A)
10 showed $\text{MH}^+=380$. UV/MS(%)=100/100.

Example 95 – *N*-((4-methylphenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-2-(4-nitrophenyl)acetamide hydrochloride(57MBT70-7D)

The title compound was prepared according to example MBT04. Yield: 28 mg (27%). $R_f=0.27$ (10% MeOH in CH_2Cl_2). HPLC-MS (method B) showed $\text{MH}^+=382$.
15 UV/MS(%)=100/100.

Example 96 – *N*-((4-methylphenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-2-(4-methoxy-3-methylphenyl)acetamide hydrochloride(57MBT70-8D)

The title compound was prepared according to example MBT04. Yield: 34 mg (32%). $R_f=0.30$ (10% MeOH in CH_2Cl_2). HPLC-MS (method B) showed $\text{MH}^+=381$.
20 UV/MS(%)=100/99.

Example 97 – *N*-((4-methylphenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-2-(4-pyridyl)acetamide hydrochloride(57MBT70-9F)

The title compound was prepared according to example MBT04. Yield: 18 mg (17%). $R_f=0.09$ (10% MeOH in CH_2Cl_2). HPLC-MS (method A) showed $\text{MH}^+=338$.
25 UV/MS(%)=100/100.

Example 98 – *N*-((4-methylphenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-2-(4-methylphenyl)acetamide hydrochloride(57MBT62B)

The title compound was prepared according to example MBT04. Yield: 10 mg (35%). $R_f=$ (10% MeOH in CH_2Cl_2). HPLC-MS (method A) showed $\text{MH}^+=351$.
30 UV/MS(%)=100/100.

Example 99 – *N*-((4-(hydroxymethyl)phenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-2-(4-methoxyphenyl)acetamide hydrochloride(57MBT72D)

To a stirred suspension of LiAlH_4 (285 mg, 7.52 mmol) in diethylether (10 mL) at 0 °C was added a solution of 4-cyanobenzyl alcohol (0.5 g, 3.76 mmol) in diethylether (5 mL) over 15 min. The grey reaction mixture was heated to reflux for 3 h. After cooling to r.t., the mixture was treated successively with water (1 mL), 2M NaOH (2 mL) and water (2 mL) under vigorous stirring. The resulting white slurry was filtered and washed with CH_2Cl_2 (20 mL). Extraction with additional CH_2Cl_2 (20 mL) and n-butanol (20 mL) and evaporation yielded an oil, which upon flash chromatography (0-15% MeOH in CH_2Cl_2) gave 152 mg (29%) of 4-(aminomethyl)benzylalcohol (**57MBT52B**) as a white solid. $R_f=0.51$ (30% MeOH in CH_2Cl_2 + 3.5% NH_4OH).

1-Methyl-4-piperidone (84 μL , 0.73 mmol) was dissolved in methanol (5 mL) and 4-(aminomethyl)benzylalcohol (**57MBT52B**) (100 mg, 0.73 mmol) was added followed by acetic acid (125 μL). NaBH_3CN (92 mg, 1.46 mmol) was added and the mixture was stirred for 3 h. The reaction mixture was evaporated and 2M NaOH (5 mL) was added. Extraction with CH_2Cl_2 (4*5 mL), drying with Na_2SO_4 and evaporation gave 152 mg (87%) of *N*-((4-(hydroxymethyl)phenyl)methyl)-4-amino-1-methylpiperidine (**57MBT56D**) as a white solid. HPLC-MS (method B) showed $\text{MH}^+=235$. UV/MS(%)=100/100.

N-((4-(Hydroxymethyl)phenyl)methyl)-4-amino-1-methylpiperidine (**57MBT56D**) (20 mg, 0.0853 mmol) was dissolved in CH_2Cl_2 (2 mL) and 4-methoxyphenylacetyl chloride (26 μL , 0.171 mmol) was added dropwise. The reaction mixture was stirred for 1 h and water (500 μL) was added followed by evaporation. A solution of sodium (5 mg, 0.179 mmol) in methanol (2 mL) was added. After stirring for 4 h, the solution was transferred to a prewashed (methanol) ion exchange column (0.88 mmol/g, 1g) and washed with methanol (4*4 mL). The remaining product was eluted off the column with 10% NH_4OH in methanol (2*4 mL) and evaporated. The resulting oil was filtered through silica (H=4 cm, D=1 cm) with methanol/ CH_2Cl_2 2:8 (20 mL), evaporated and subjected to a second ion exchange column (0.88 mmol/g, 1g). The column was washed with methanol (8*4 mL) and the remaining product was eluted off the column with 10% NH_4OH in

methanol (2*4 mL) and evaporated on rotavap and oilpump. The product was dissolved in CH₂Cl₂ (0.5 mL) and HCl in diethylether (1.0 M, 0.1 mL, 0.1 mmol) was added. The solution was added to n-heptane (3 mL) and evaporation afforded 14 mg (39%) of the title compound as a white solid. $R_f=0.16$ (10% MeOH in CH₂Cl₂).

- 5 HPLC-MS (method B) showed $MH^+=383$. UV/MS(%)=100/96.

Example 100 - 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-isopropylpiperidin-4-yl)acetamide (47AKU-7)

1-Trifluoroacetyl-4-piperidone (47AKU-2)

- 4-Piperidone hydrochloride monohydrate (3.85 g, 25 mmol) and
10 Triethylamine (10.5 ml, 75 mmol) were partly dissolved in 100 ml of dichloromethane and stirred for 10 min. Reaction mixture was then cooled on ice-bath and trifluoroacetic anhydride (7.2 ml, 50 mmol) was slowly added over 10 min. Ice-bath was removed and mixture was stirred overnight. Additional trifluoroacetic anhydride (2 ml) was added and the mixture was stirred for 1 hr. Water (200ml) was
15 added. Phases were separated and aq. phase was re-extracted with dichloromethane. Combined organic phases were washed with brine, dried over MgSO₄ and concentrated (40°C) giving 4.97 g (100%) 47AKU-2 as yellow crystals. TLC (5% methanol in dichloromethane): $R_f = 0.8$. ¹H-NMR (400MHz, CDCl₃): $\delta = 3.87$ -3.99 (4H, m); 2.54-2.61 (4H, m). ¹³C-NMR (CDCl₃): $\delta = 204.7, 118.0, 115.1, 44.2, 42.8,$
20 41.2, 40.5.

4-(4-Methylbenzylamino)-1-trifluoroacetyl-piperidine (47AKU-3)

- 47AKU-2 (4.97 g, 25 mmol) was dissolved in 100 ml methanol and 4-methylbenzyl-amine (3.2 ml, 25 mmol) was added. Mixture was stirred and acetic acid (~2 ml) was added until pH~5. NaCNBH₃ (3.15 g, 50 mmol) was slowly added.
25 After magnetic stirring for 20 hrs the methanol was partly removed on the rotary evaporator (40°C). Dichloromethane, 2M NaOH and water were added until pH~10. Phases were separated and aq. phase was then re-extracted twice with dichloromethane. Combined organic phases were washed with brine and dried over MgSO₄. Concentration(40°C) yielded 6.94 g (92%) 47AKU-3. TLC (10% methanol in dichloromethane): $R_f = 0.6$. HPLC-MS (Method A): $M^+ = 301.0$
30 (UV/MS(%)=94/100).

2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-trifluoroacetyl-piperidin-4-yl)acetamide (47AKU-4)

47AKU-3 (3.01 g, 10 mmol) in 25 ml of dichloromethane was placed in a 100 ml flask. Triethylamine (1.4 ml, 10 mmol) was added and the mixture was cooled on an ice-bath and stirred for 10 min. 4-Chlorophenylacetyl chloride (1.90 g, 10 mmol) was dissolved in 10 ml dichloromethane and added slowly to the ice-cold mixture.

5 After 15 min. the ice-bath was removed and the mixture was left for 1 hr. Precipitation was observed. The reaction mixture was then concentrated at aspirator pressure (40°C). The crude product was purified by flash chromatography (0-50% ethylacetate in heptane) yielding 2.38 g (53%) **47AKU-4**. TLC (100% dichloromethane): $R_f = 0.6$. HPLC-MS (Method A): $M^+ = 453.0$ (UV/MS(%)=89/84).

10 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(piperidin-4-yl)acetamide (**47AKU-6**)

47AKU-4 (2.38 g; ~5 mmol) was dissolved in 50 ml of methanol. K_2CO_3 (3.5 g; 25 mmol) was added in one portion. After magnetic stirring for 20 hrs, additional K_2CO_3 (1 g) was added. After 4 hrs magnetic stirring methanol was partly removed
15 by evaporation (40°C). Ethyl acetate (100 ml) and water (100 ml) were added. The phases were separated and the aq. phase was then re-extracted with ethylacetate. The combined organic phases were dried over $MgSO_4$ and concentrated (40°C) giving 1.95 g (100%) **47AKU-6**. TLC (20% methanol in dichloromethane): $R_f = 0.3$. HPLC-MS (Method A): $M^+ = 357.1$ (UV/MS(%)=84/95).

20 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-isopropylpiperidin-4-yl)-acetamide (**47AKU-7**)

47AKU-6 (358 mg, 1.0 mmol) was dissolved in 20 ml of acetonitrile. Triethylamine (1.4 ml, 10 mmol) was added and mixture was stirred for 10 min. Isopropyl bromide (370 mg, 3.0 mmol) was dissolved in 5 ml of acetonitrile and
25 added to the reaction mixture which was stirred at room temp. for 20 hrs and then heated to 60°C for 4 hrs. After cooling, ethylacetate (25 ml) and water (25 ml) were added. The phases were separated and the aq. phase was then re-extracted with ethylacetate. The combined organic phases were washed with brine, dried over $MgSO_4$ and concentrated (40°C) giving 362 mg of crude product. Purification by flash
30 chromatography (0-10% methanol in dichloromethane) and HCl-precipitation from 2M HCl/diethyl ether in dichloromethane/heptane gave 76 mg (18%) **47AKU-7**. TLC (10% methanol in dichloromethane): $R_f = 0.4$. Mp = 223-224°C. HPLC-MS (Method A): $M^+ = 399.1$ (UV/MS(%)=100/99). 1H -NMR (400 MHz, $CDCl_3$): $\delta = 7.03$ -7.29

(8H, m); 4.86 (1H, m); 4.61 (2H, m); 3.58 (2H, m); 3.37 (3H, m); 2.82 (2H, m); 2.64 (2H, m); 2.34 (3H, s); 1.80 (2H, m); 1.39 (6H, d). ¹³C-NMR (CDCl₃): δ= 172.4, 137.4, 134.8, 133.3, 133.1, 130.4, 129.9, 129.0, 125.8, 58.0, 49.5, 48.2, 46.6, 40.4, 26.0, 21.2, 17.0.

5 **Example 101 - 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-ethylpiperidin-4-yl)acetamide (47AKU-12)**

47AKU-6 (358 mg, 1.0 mmol) was dissolved in 20 ml of acetonitrile. Triethylamine (1.4 ml, 10 mmol) was added and the mixture was stirred for 10 min. Ethyl bromide (370 µl, 5.0 mmol) was added. The mixture was then heated to 50°C and stirred overnight. After cooling, water (25 ml) and ethylacetate (25 ml) were
10 added. The phases were separated and the aq. phase was re-extracted with ethylacetate. The combined organic phases were washed with brine and dried over MgSO₄. Evaporation(40°C) yielded 406 mg of crude product. Purification by ion exchange chromatography (washout with 10% aq. NH₄OH (25%) in methanol) gave
15 166 mg (43%) 47AKU-12. The HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.5. HPLC-MS (Method A): M⁺ = 385.1 (UV/MS(%)=100/99). ¹H-NMR (400 MHz, CDCl₃, rotamers): δ= 7.02-7.34 (8H, m); 4.62 (1H, m); 4.46 and 4.53 (2H, 2s); 3.81 (1H, s); 3.55 (2H, s); 2.92 (2H, m); 2.34 (3H, s); 2.29 (1H, s); 1.98 (2H, m); 1.52-1.84 (4H,
20 m); 1.03 (3H, t). ¹³C-NMR (CDCl₃): δ= 171.7, 137.2, 135.4, 133.9, 132.8, 130.4, 129.7, 128.9, 125.8, 52.8, 52.4, 46.5, 40.8, 31.2, 29.8, 21.2, 12.4.

Example 102 - 2-Phenyl-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide (47AKU-13)

47AKU-5 (218mg, 1.0 mmol) was dissolved in 2 ml of dichloromethane in a
25 50 ml flask. Phenylacetyl chloride (134 µl, 1.0 mmol) was added. After 3 hrs stirring at room temp. mixture was concentrated on Rotavapor (40°C). Crude product was purified by ion exchange chromatography (washout with 10% aq. NH₄OH (25%) in methanol) and flash chromatography (0-10% methanol in dichloromethane) giving 48 mg (14%) 47AKU-13. HCl-salt was prepared from 2M HCl/diethylether in
30 dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.4. HPLC-MS (Method A): M⁺ = 337.1 (UV/MS(%)=98/98). ¹H-NMR (400 MHz, CDCl₃, rotamers): δ= 7.01-7.40 (9H, m); 4.63 (1H, m); 4.53 and 4.45 (2H, 2s); 3.85 and 3.61 (2H, 2s); 2.86 and 2.77 (2H, 2m); 2.35 and 2.29 (3H, 2s); 2.25 and 2.20 (3H, 2s); 2.09

(2H, m); 1.61-1.86 (4H, m). ^{13}C -NMR (CDCl_3): δ = 172.2, 137.1, 135.5, 129.7, 128.9, 128.8, 127.2, 126.9, 125.8, 55.3, 51.6, 46.6, 46.1, 41.6, 29.5, 21.2.

Example 103 - 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide (47AKU-8)

5 **4-(4-Methylbenzylamino)-1-methyl-piperidine (47AKU-5)**

1-Methyl-4-piperidone (1.13 g, 10 mmol) was dissolved in 20 ml of methanol and added to a 100 ml flask. 4-Methylbenzylamine (1.21 g, 10 mmol) in 10 ml of methanol was added. Acetic acid (~1.5 ml) was added until pH~5. NaCNBH_3 (1.26 g, 20 mmol) was slowly added. After 20 hrs magnetic stirring methanol was partly
10 removed on Rotavapor (40°C). Dichloromethane, water and 2M NaOH were added until pH~10. The phases were separated and aq. phase was extracted twice with dichloromethane. The combined organic phases were washed with brine and dried over MgSO_4 . Concentration on Rotavapor (40°C) yielded 2.06 g crude (93%)
47AKU-5. TLC (20% methanol in dichloromethane): R_f = 0.3. HPLC-MS (Method
15 A): M^+ = 219.1 (UV/MS(%)=89/98).

2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-
acetamide

(47AKU-8)

47AKU-5 (437 mg, 2.0 mmol) was dissolved in 10 ml of dichloromethane in a
20 50 ml flask. Triethylamine (280 μl , 2.0 mmol) was added and the mixture was cooled to 0°C on an ice bath and stirred for 10 min. 4-Chlorophenylacetyl chloride (380 mg, 2.0 mmol) was dissolved in 10 ml of dichloromethane and added to the cooled mixture. After 2 hrs stirring at room temp. additional dichloromethane (10 ml) and water (20 ml) were added. The phases were separated and the aq. phase was re-
25 extracted with dichloromethane. The combined organic phases were dried over MgSO_4 and concentrated on the Rotavapor (40°C) giving 755 mg of crude product. Purification by flash chromatography (0-10% methanol in dichloromethane) gave 485 mg (65%) product. Further purification by ion exchange chromatography (washout with 10% aq. NH_4OH (25%) in methanol) gave 239 mg (32%) **47AKU-8**. The HCl-
30 salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.4. Mp = 217-219°C. HPLC-MS (Method A): M^+ = 371.1 (UV/MS(%)=99/99). ^1H -NMR (400 MHz, CD_3OD): δ = 7.05-7.39 (8H, m); 4.80 (3H, s); 4.62 + 4.56 (2H, 2s); 4.35 (1H, m); 4.00 (1H, s); 3.71 (1H, s); 3.46

(2H, m); 3.06 (2H, m); 2.80 (3H, s); 2.32 + 2.27 (3H, 2s); 2.19 (1H, m). ¹³C-NMR (CD₃OD): δ= 173.0, 137.5, 134.5, 133.9, 132.6, 130.6, 129.5, 128.5, 126.2, 54.0, 51.4, 42.6, 40.2, 31.8, 26.6, 19.9.

Example 104 - 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-cyclopentylpiperidin-4-yl)-acetamide (47AKU-11)

47AKU-6 (358 mg, 1.0 mmol) was dissolved in 20 ml of acetonitrile. Triethylamine (1.4 ml, 10 mmol) was added and mixture was stirred for 10 min. Cyclopentylbromide (540 μl, 5.0 mmol) was added and the mixture was stirred at room temp. After 20 hrs the mixture was heated to 50°C for an additional 24 hrs. The reaction mixture was then cooled and water (25 ml) and ethylacetate (25 ml) were added. The phases were separated and the aq. phase was re-extracted with ethylacetate. The combined organic phases were washed with brine and dried over MgSO₄. Concentration on Rotavapor (45°C) yielded 426 mg of crude product. Purification by ion exchange chromatography (washout with 10% aq. NH₄OH (25%) in methanol) and flash chromatography (0-10% methanol in dichloromethane) gave 76 mg (18%) **47AKU-11**. The HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.5. HPLC-MS (Method A): M⁺ = 425.1 (UV/MS(%)=100/97). ¹H-NMR (400 MHz, CDCl₃, rotamers): δ= 7.01-7.34 (8H, m); 4.67 (1H, m); 4.49 and 4.52 (2H, 2s); 3.54 (2H, s); 3.15 and 3.02 (2H, 2m); 2.64 (1H, m); 2.27 and 2.34 (3H, 2s); 2.20 (1H, m); 1.85 (4H, m); 1.69 (4H, m); 1.53 (4H, m); 1.37 (1H, m). ¹³C-NMR (CDCl₃): δ= 171.9, 137.2, 135.2, 133.8, 132.9, 130.4, 129.7, 128.9, 125.8, 67.7, 52.4, 52.1, 46.5, 40.7, 30.2, 28.8, 24.3, 21.2.

Example 105 - 2-(4-Fluorophenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide (47AKU-14)

47AKU-5 (218mg, 1.0 mmol) was dissolved in 3 ml of dichloromethane in a 50 ml flask. 4-Fluorophenylacetyl chloride (150 μl, 1.1 mmol) was added. After 4 hrs stirring at room temp. the mixture was concentrated on Rotavapor (40°C). The crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 243 mg (68%) **47AKU-14**. The HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.5. HPLC-MS (Method A): M⁺ = 355.1 (UV/MS(%)=100/100). ¹H-NMR (400 MHz, CDCl₃): δ= 6.92-7.33 (8H, m); 4.73

(1H, m); 4.52 (2H, s); 3.56 (2H, 2s); 3.44 (5H, m); 3.25 (2H, m); 2.52-2.67 (4H, m); 2.33 (3H, s). ¹³C-NMR (CDCl₃): δ= 172.5, 163.3, 160.9, 139.5, 134.8, 130.6, 129.8, 125.8, 115.8, 54.6, 50.8, 49.9, 46.7, 40.4, 27.2, 21.2.

Example 106 - 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-(2-hydroxyethyl)-piperidin-4-yl)-acetamide (47AKU-18)

47AKU-6-2 (358 mg, 1.0 mmol) was dissolved in 10 ml of acetonitrile in 50 ml flask. Triethylamine (1.4 ml, 10 mmol) was added and mixture was stirred for 10 min.

2-Bromoethanol (215 µl, 3.0 mmol) was added. Reaction mixture was then heated to 60°C and stirred overnight. After cooling ethylacetate (25 ml) and water (25 ml) were added. Phases were separated and aq. phase was re-extracted with ethylacetate. Combined organic phases were washed with brine, dried over MgSO₄ and concentrated on Rotavapor (40°C) giving 406 mg crude product. Purification by flash chromatography (0-10% methanol in dichloromethane) afforded 253 mg (63%) **47AKU-18**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.4. HPLC-MS (Method A): M⁺ = 401.1 (UV/MS(%)=100/100). ¹H-NMR (400 MHz, CDCl₃, rotamers): δ= 7.04-7.34 (8H, m); 4.60 (1H, m); 4.52 and 4.45 (2H, 2s); 3.55 (4H, m); 3.03 (1H, bs); 2.92 (2H, m); 2.52 (2H, m); 2.36 and 2.31 (3H, 2s); 2.19 (2H, m); 1.66 (4H, m). ¹³C-NMR (CDCl₃): δ= 171.7, 137.3, 135.2, 133.8, 132.9, 130.4, 129.8, 128.9, 125.8, 59.4, 58.1, 53.1, 52.3, 46.8, 40.8, 29.7, 21.2.

Example 107 - 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-cyclobutylpiperidin-4-yl)-acetamide (47AKU-19)

1-Cyclobutyl-4-piperidone (47AKU-15)

Partly dissolved quaternary salt (1.23 g, 3.7 mmol) (prepared according to the procedure outlined in the synthesis of **47AKU-47**) was slowly added to a refluxing solution of Cyclobutylamine (178 mg, 2.5 mmol) and Potassium carbonate (48 mg, 0.34 mmol) in ethanol. The mixture was refluxed for 1.5 hrs. After cooling to room temp. water (10 ml) and dichloromethane (25 ml) were added. Phases were separated and aq. phase was re-extracted with dichloromethane. Combined organic phases were dried over MgSO₄ and concentrated on Rotavapor (40°C) giving 419 mg crude **47AKU-15**. TLC (10% methanol in dichloromethane): R_f = 0.4. HPLC-MS (Method A): M⁺ = 154.1 (MS(%)=75).

4-(4-Methylbenzylamino)-1-cyclobutyl-piperidine (47AKU-16)

4-Methylbenzylamine (215 mg, 1.8 mmol) was dissolved in 5 ml methanol and placed in 50 ml flask. **47AKU-15** (270 mg, 1.8 mmol) in 5 ml methanol was added. Acetic acid (0.3 ml) was added until pH~5. NaCNBH₃ (226 mg, 3.6 mmol) was slowly added. Gas evolution observed. After 24 hrs magnetic stirring dichloromethane, 2M NaOH and water were added until pH~10. Phases were separated and aq. phase was then re-extracted with dichloromethane. Combined organic phases were dried over MgSO₄ and concentrated on Rotavapor (40°C) yielding 419 mg crude **47AKU-16**. TLC (10% methanol in dichloromethane): R_f = 0.3. HPLC-MS (Method A): M⁺ = 259.1 (UV/MS(%))=44/87).

2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-cyclobutylpiperidin-4-yl)-acetamide (47AKU-19)

47AKU-16 (209 mg, 0.8 mmol) was placed in 50 ml flask and 5 ml dichloromethane was added. 4-Chlorophenylacetyl chloride (171 mg, 0.9 mmol) in 5 ml dichloromethane was added. After 5 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 101 mg (31%) product. Further purification by ion exchange chromatography (washout with 10% aq. NH₄OH (25%) in methanol) gave 55 mg (17%) **47AKU-19**. Oxalate-salt was prepared from Oxalic acid (1.1 eq) in dichloromethane/ heptane. TLC (10% methanol in dichloromethane): R_f = 0.6. HPLC-MS (Method B): M⁺ = 411.2 (UV/MS(%))=91/86). ¹H-NMR (400 MHz, CDCl₃, rotamers): δ = 7.33-7.01 (8H, m); 4.62 (1H, m); 4.52 and 4.46 (2H, 2s); 3.80 (1H, s); 3.45 and 3.54 (2H, 2s); 2.86 (2H, m); 2.66 (2H, m); 2.28 and 2.34 (3H, 2s); 1.98 (2H, m); 1.80 (2H, m); 1.70-1.52 (6H, m). ¹³C-NMR (CDCl₃): δ = 171.7, 137.2, 135.4, 133.9, 132.9, 130.4, 129.7, 128.9, 125.7, 60.4, 52.3, 49.4, 46.5, 40.7, 29.4, 27.6, 21.2, 14.2.

Example 108 - 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-cyclobutylpiperidin-4-yl)acetamide (47AKU-20)

47AKU-16 (209 mg, 0.8 mmol) was placed in 50 ml flask and 5 ml dichloromethane was added. 4-Methoxyphenylacetyl chloride (167 mg, 0.9 mmol) in 5 ml dichloromethane was added. After 5 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 72 mg (22%) product.

Further purification by ion exchange chromatography (washout with 10% aq. NH_4OH (25%) in methanol) gave 67 mg (20%) **47AKU-20**. Oxalate-salt was prepared from Oxalic acid (1.1 eq) in dichloromethane/heptane. TLC (10% methanol in dichloromethane): $R_f = 0.6$. HPLC-MS (Method B): $M^+ = 407.3$ (UV/MS(%)=93/77).

5 $^1\text{H-NMR}$ (400 MHz, CDCl_3 , rotamers): $\delta = 7.26\text{--}6.79$ (8H, m); 4.62 (1H, m); 4.52 and 4.45 (2H, 2s); 3.79 (1H, m); 3.77 (3H, s); 3.52 and 3.45 (2H, 2s); 2.84 (2H, m); 2.66 (2H, m); 2.34 and 2.28 (3H, 2s); 1.98 (2H, m); 1.81 (2H, m); 1.72-1.51 (6H, m). $^{13}\text{C-NMR}$ (CDCl_3): $\delta = 172.5, 158.7, 137.0, 135.7, 130.4, 129.8, 127.4, 125.8, 114.3, 60.4, 55.5, 52.1, 49.4, 46.4, 40.6, 29.4, 27.6, 21.2, 14.2$.

10 **Example 109 - (47AKU-21) 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(tropin-4-yl)acetamide (47AKU-21)**

4-(4-Methylbenzylamino)-tropane (47AKU-17)

4-Methylbenzylamine (607 mg, 5.0 mmol) was dissolved in 10 ml methanol and placed in 100 ml flask. Tropinone (697 mg, 5.0 mmol) in 10 ml methanol was added. Acetic acid (0.75 ml) was added until pH~5. NaCNBH_3 (628 mg, 10 mmol) was slowly added. Gas evolution observed. After 20 hrs magnetic stirring dichloromethane, 2M NaOH and water were added until pH~10. Phases were separated and aq. phase was then re-extracted with dichloromethane. Combined organic phases were dried over MgSO_4 . Concentration on Rotavapor (40°C) yielded

15 1.14 g crude **47AKU-17**. TLC (10% methanol in dichloromethane): $R_f = 0.4$. HPLC-MS (Method A): $M^+ = 245.2$ (UV/MS(%)=65/96).

20 **2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(tropin-4-yl)-acetamide (47AKU-21)**

47AKU-17 (244 mg, 1.0 mmol) was placed in 50 ml flask and 5 ml dichloromethane was added. 4-Methoxyphenylacetyl chloride (203 mg, 1.1 mmol) in 10 ml dichloromethane was added. After 3 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by ion exchange chromatography (washout with 10% aq. NH_4OH (25%) in methanol) and flash chromatography (0-10% methanol in dichloromethane) giving 202 mg (51%)

25 **47AKU-21**. Oxalate-salt was prepared from Oxalic acid (1.1 eq) in dichloromethane/heptane. TLC (10% methanol in dichloromethane): $R_f = 0.4$. HPLC-MS (Method B): $M^+ = 393.3$ (UV/MS(%)=94/92). $^1\text{H-NMR}$ (400 MHz, CDCl_3 , isomers): $\delta = 7.02\text{--}7.17$ (6H, m); 6.78-6.87 (2H, m); 4.74 (1H, s); 4.44 (1H, s); 3.78

and 3.77 (3H, 2s); 3.68 (1H, m); 3.66 and 3.55 (3H, 2s); 2.65 (2H, m); 2.56 (2H, m); 2.32 (3H, s); 2.12-2.26 (6H, m); 2.05 (2H, m). ^{13}C -NMR (CDCl_3): δ = 173.2, 171.4, 158.8, 137.1, 129.7, 127.6, 126.9, 126.0, 114.4, 63.4, 60.9, 55.5, 54.6, 47.5, 41.5, 40.4, 32.8, 31.1, 27.5, 24.9, 21.2.

5 **Example 110 - *N*-(4-Methylbenzyl)-*N*-(1-methylpiperidin-4-yl)-*N'*-benzyl-carbamide (47AKU-22)**

47AKU-5 (219 mg, 1.0 mmol) was dissolved in 5 ml dichloromethane and placed in 50 ml flask. Benzylisocyanate (160 mg, 1.2 mmol) in 5 ml dichloromethane was added. After 16 hrs magnetic stirring the reaction mixture was concentrated on
10 Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 236 mg (67%) 47AKU-22. Oxalate-salt was prepared from Oxalic acid (1.1 eq) in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.5. HPLC-MS (Method B): M^+ = 352.3 (UV/MS(%)=100/100). ^1H -NMR (400 MHz, CDCl_3): δ = 7.26-7.02 (9H, m); 4.61 (1H, m); 4.41 (1H, m); 4.33 (4H, m); 2.87 (2H, m); 2.32 (3H, s); 2.25 (3H, s); 2.09 (2H, m); 1.79-1.62 (4H, m). ^{13}C -NMR (CDCl_3): δ = 158.6, 139.7, 137.3, 135.4, 129.8, 128.6, 127.4, 127.2, 126.2, 55.5, 52.2, 46.2, 45.8, 45.0, 30.2, 21.2.

15 **Example 111 - *N*-(4-Methylbenzyl)-*N*-(1-methylpiperidin-4-yl)-*N'*-phenyl carbamide (47AKU-24)**

20 47AKU-5 (219 mg, 1.0 mmol) was dissolved in 5 ml dichloromethane and placed in 50 ml flask. Phenylisocyanate (143 mg, 1.2 mmol) in 5 ml dichloromethane was added. After 4 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 181 mg (54%) 47AKU-24. HCl-salt was
25 prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.4. HPLC-MS (Method A): M^+ = 338.3 (UV/MS(%)=100/100). ^1H -NMR (400 MHz, CDCl_3): δ = 7.12-7.24 (8H, m); 6.93-6.98 (1H, m); 6.26 (1H, s); 4.45 (3H, s); 2.90 (2H, d); 2.36 (3H, s); 2.28 (3H, s); 2.12 (2H, m); 1.69-1.85 (4H, m). ^{13}C -NMR (CDCl_3): δ = 156.1, 139.3, 137.8, 134.9, 130.1, 128.9, 126.3, 123.1, 119.9, 55.5, 52.3, 46.3, 46.2, 30.3, 21.3.
30

Example 112 - *N*-Phenethyl-*N*-(1-methylpiperidin-4-yl)-*N'*-benzyl-carbamide (47AKU-25)

4-(2-Phenylethyl)amino-1-methylpiperidine (110 mg, 0.5 mmol) was dissolved in 5 ml dichloromethane and placed in 50 ml flask. Benzylisocyanate (80 mg, 0.6 mmol) in 5 ml dichloromethane was added. After 20 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 164 mg (84%) **47AKU-25**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.4. HPLC-MS (Method A): M^+ = 352.3 (UV/MS(%)=100/100). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ = 7.34-7.09 (10H, m); 4.52 (1H, m); 4.35 (2H, d); 4.08 (1H, m); 3.33 (2H, t); 2.92 (2H, m); 2.82 (2H, t); 2.28 (3H, s); 2.07 (2H, m); 1.84-1.66 (4H, m). $^{13}\text{C-NMR}$ (CDCl_3): δ = 157.9, 139.8, 139.1, 129.0, 128.9, 128.8, 127.8, 127.4, 126.9, 55.7, 52.8, 46.2, 45.3, 44.8, 37.5, 30.6.

Example 113 - 2-Phenyl-*N*-(4-methoxybenzyl)-*N*-(1-methylpiperidin-4-yl)-acetamide (47AKU-26a)

50ELH-18 (118 mg, 0.5 mmol) was dissolved in 5 ml dichloromethane in 50 ml flask.

4-Fluorophenylacetyl chloride (104 mg, 0.6 mmol) was added. After 20 hrs stirring at room temp. mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 87 mg (49%) **47AKU-26a**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/

heptane. HPLC-MS (Method A): M^+ = 353.1 (UV/MS(%)=96/88).

Example 114 - 2-(4-Trifluoromethylphenyl)-*N*-(4-methoxybenzyl)-*N*-(1-methylpiperidin-4-yl)-acetamide (47AKU-26b)

50ELH-18 (118 mg, 0.5 mmol) was dissolved in 5 ml dichloromethane in 50 ml flask.

4-Trifluoromethylphenylacetyl chloride (134 mg, 0.6 mmol) was added. After 20 hrs stirring at room temp. mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 81 mg (39%) **47AKU-26b**. HCl-salt was prepared from 2M HCl/diethylether

in dichloromethane/heptane. HPLC-MS (Method A): M^+ = 421.1 (UV/MS(%)=90/100).

Example 115 - 2-(4-Fluorophenyl)-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-acetamide (47AKU-26c)

5 **50ELH-18** (118 mg, 0.5 mmol) was dissolved in 5 ml dichloromethane in 50 ml flask.

4-Fluorophenylacetyl chloride (104 mg, 0.6 mmol) was added. After 20 hrs stirring at room temp. mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving
10 68 mg (37%) **47AKU-26c**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. HPLC-MS (Method A): M^+ = 371.1 (UV/MS(%)=100/97).

Example 116 - 2-(4-Methoxyphenyl)-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-acetamide (47AKU-26d)

15 **50ELH-18** (118 mg, 0.5 mmol) was dissolved in 5 ml dichloromethane in 50 ml flask.

4-Methoxyphenylacetyl chloride (111 mg, 0.6 mmol) was added. After 20 hrs stirring at room temp. mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving
20 77 mg (40%) **47AKU-26d**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. HPLC-MS (Method A): M^+ = 383.1 (UV/MS(%)=100/100).

Example 117 - 2-(4-Methylphenyl)-N-(4-chlorobenzyl)-N-(1-methylpiperidin-4-yl)-acetamide (47AKU-28)

4-(4-Chlorobenzylamino)-1-methyl-piperidine (**47AKU-27**)

1-Methyl-4-piperidone (566 mg, 5.0 mmol) was dissolved in 10 ml methanol
25 and placed in 100 ml flask. 4-Chlorobenzylamine (708 mg, 5.0 mmol) was added. Mixture was stirred and Acetic acid (~0.75 ml) was added until pH~5. NaCNBH₃ (628 mg, 10 mmol) was slowly added. Gas evolution observed. After magnetic stirring for 16 hrs methanol was partly removed on Rotavapor (40°C).
Dichloromethane, 2M NaOH and water were added until pH~10. Phases were
30 separated and aq. phase was then re-extracted with dichloromethane. Combined organic phases were dried over MgSO₄. Concentration on Rotavapor (40°C) yielded 1.14 g crude **47AKU-27**. TLC (10% methanol in dichloromethane): R_f = 0.3. HPLC-MS (Method A): M^+ = 239.1 (MS(%)=96).

2-(4-Methylphenyl)-N-(4-chlorobenzyl)-N-(1-methylpiperidin-4-yl)-
acetamide

(47AKU-28)

p-Tolylacetic acid (1.50 g) was dissolved in 10 ml thionylchloride and placed
5 in 50 ml flask. Mixture was heated to reflux for 2 hrs and then concentrated on
Rotavapor (40°C).

p-Tolylacetic chloride (202 mg, 1.2 mmol) in 5 ml dichloromethane was
added to **47AKU-27** (239 mg, 1.0 mmol) in 5 ml dichloromethane. After 4 hrs
magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude
10 product was purified by flash chromatography (0-10% methanol in dichloromethane)
giving 104 mg (28%) **47AKU-28**. HCl-salt was prepared from 2M HCl/diethylether
in dichloromethane/

heptane. TLC (10% methanol in dichloromethane): $R_f = 0.5$. HPLC-MS
(Method A): $M^+ = 371.1$ (UV/MS(%)=100/90). $^1\text{H-NMR}$ (400 MHz, CDCl_3 ,
15 rotamers): $\delta = 7.34\text{--}6.99$ (8H, m); 4.57 (1H, m); 4.50 and 4.44 (2H, 2s); 3.80 (1H, s);
3.55 (1H, s); 2.96 and 2.82 (2H, 2m); 2.34 (1H, m); 2.32 (3H, s); 2.24 and 2.15 (3H,
2s); 1.91 (1H, m); 1.81-1.59 (4H, m). $^{13}\text{C-NMR}$ (CDCl_3): $\delta = 172.5, 138.2, 136.8,$
133.4, 131.8, 129.7, 129.2, 128.6, 127.4, 54.9, 51.3, 46.7, 41.3, 30.6, 28.6, 21.2.

**Example 118 - 2-(4-Hydroxyphenyl)-N-(4-methylbenzyl)-N-(1-
20 methylpiperidin-4-yl)-acetamide (47AKU-29)**

42ELH-77 (41 mg, 0.1 mmol) was dissolved in 1 ml dry dichloromethane and
placed in oven-dried 10 ml flask. Mixture was cooled to -78°C on a dry-
ice/isopropanol bath. Borontribromide (1.0 M in dichloromethane, 150 μl , 0.15 mmol)
was slowly added at

25 -78°C. Ice-bath was removed and mixture was left at room temp. for 2 hrs.
Water (3 ml) and saturated NaCl (aq.) were added and aq. phase was extracted with
dichloromethane, ethylacetate and n-butanol. Combined organic phases were dried
over MgSO_4 and concentrated on Rotavapor (40°C). Crude product was purified by
flash chromatography (0-20% methanol in dichloromethane) giving 22 mg (63%)
30 **47AKU-29**. HCl-salt was prepared from 2M HCl/diethylether in
dichloromethane/heptane. TLC (10% methanol in dichloromethane): $R_f = 0.3$. HPLC-
MS (Method A): $M^+ = 353.2$ (UV/MS(%)=100/100). $^1\text{H-NMR}$ (400 MHz, CDCl_3 ,
rotamers): $\delta = 7.07\text{--}6.60$ (8H, m); 4.48 (1H, m); 4.39 (2H, s); 3.76 and 3.66 (4H, 2bs);

3.41 (2H, s); 3.08 (2H, m); 2.49 (1H, m); 2.42 (2H, bs); 2.22 and 2.16 (3H, 2s); 1.96-1.82 (2H, m); 1.66-1.56 (1H, m). ^{13}C -NMR (CDCl_3): δ = 173.7, 156.0, 137.3, 134.6, 129.7, 129.6, 125.7, 125.4, 115.7, 54.4, 50.4, 46.8, 44.0, 40.5, 27.3, 20.9.

Example 119 - *N*-Phenethyl-*N*-(1-methylpiperidin-4-yl)-*N'*-phenyl-carbamide (47AKU-30)

4-(2-Phenylethyl)amino-1-methylpiperidine (110 mg, 0.5 mmol) was dissolved in 5 ml dichloromethane and placed in 50 ml flask. Phenylisocyanate (71 mg, 0.6 mmol) in 5 ml dichloromethane was added. After 16 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified
10 twice by flash chromatography (0-10% methanol in dichloromethane) giving 131 mg (78%) **47AKU-30**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.4. HPLC-MS (Method A): M^+ = 338.1 (UV/MS(%)=99/100). ^1H -NMR (400 MHz, CDCl_3): δ = 7.36-6.93 (10H, m); 6.24 (1H, s); 4.31 (1H, m); 3.50 (2H, t); 3.20 (2H, d); 2.89 (2H,
15 t); 2.57 (2H, m); 2.50 (3H, s); 2.26 (2H, m); 1.79 (2H, m). ^{13}C -NMR (CDCl_3): δ = 155.8, 139.2, 139.0, 129.4, 129.3, 128.9, 127.3, 123.2, 120.4, 54.9, 51.3, 45.5, 44.3, 37.6, 28.3.

Example 120 - *N*-(3-Phenylpropyl)-*N*-(1-methylpiperidin-4-yl)-*N'*-benzyl-carbamide (47AKU-31)

4-(3-Phenylpropyl)amino-1-methylpiperidine (160 mg, 0.7 mmol) was dissolved in 5 ml dichloromethane and placed in 50 ml flask. Benzylisocyanate (107 mg, 0.8 mmol) in 5 ml dichloromethane was added. After 2 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified
25 twice by flash chromatography (0-10% methanol in dichloromethane) giving 156 mg (61%) **47AKU-31**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.3. HPLC-MS (Method A): M^+ = 366.1 (UV/MS(%)=100/100). ^1H -NMR (400 MHz, CDCl_3): δ = 7.34-7.07 (10H, m); 4.33 (3H, m); 4.14 (1H, m); 3.04 (2H, m); 2.89 (2H, d); 2.57 (2H,
30 t); 2.28 (3H, s); 2.06 (2H, m); 1.87 (2H, m); 1.75-1.62 (4H, m). ^{13}C -NMR (CDCl_3): δ = 157.5, 141.0, 140.0, 129.0, 128.6, 128.3, 128.0, 127.6, 126.6, 55.6, 52.1, 46.3, 45.1, 41.6, 33.4, 32.2, 30.6.

Example 121 - *N*-(3-Phenylpropyl)-*N*-(1-methylpiperidin-4-yl)-*N'*-phenyl-carbamide (47AKU-32)

4-(3-Phenylpropyl)amino-1-methylpiperidine (160 mg, 0.7 mmol) was dissolved in 5 ml dichloromethane and placed in 50 ml flask. Phenylisocyanate (95
5 mg, 0.8 mmol) in 5 ml dichloromethane was added. After 20 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 106 mg (43%)
47AKU-32. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.3. HPLC-
10 MS (Method A): M^+ = 352.1 (UV/MS(%)=100/100). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ = 7.35-6.95 (10H, m); 5.99 (1H, s); 4.18 (1H, m); 3.17 (2H, t); 2.91 (2H, d); 2.65 (2H, t); 2.28 (3H, s); 2.07 (2H, m); 1.97 (2H, m); 1.81-1.66 (4H, m). $^{13}\text{C-NMR}$ (CDCl_3): δ = 154.9, 141.0, 139.3, 129.2, 129.0, 129.0, 126.8, 123.1, 120.0, 55.6, 52.2, 46.2, 41.8, 33.4, 32.3, 30.6.

15 **Example 122 - 2-(4-Methoxyphenyl)-2,2-ethylene-*N*-(4-methylbenzyl)-*N*-(1-methylpiperidin-4-yl) acetamide (47AKU-33)**

1-(4-Methoxyphenyl)-1-cyclopropane carboxylic acid (230 mg, 1.2 mmol) was dissolved in 2 ml thionylchloride and placed in 50 ml flask. Mixture was heated to reflux for 2 hrs and then concentrated on Rotavapor (40°C). The acid chloride (250
20 mg, 1.2 mmol) in 5 ml dichloromethane was added to **47AKU-5** (220 mg, 1.0 mmol) in 5 ml dichloromethane. After 2 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified twice by flash chromatography (0-10% methanol in dichloromethane) giving 201 mg (51%)
47AKU-33. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.6. HPLC-
25 MS (Method A): M^+ = 393.2 (UV/MS(%)=95/88).

$^1\text{H-NMR}$ (400 MHz, CDCl_3 , rotamers): δ = 7.22-6.70 (8H, m); 4.44 (2H, s); 4.26 (1H, m); 3.74 (3H, s); 3.12 and 2.89 (2H, 2m); 2.51 (1H, m); 2.32 (3H, m); 2.26 (3H, s); 2.08-1.52 (4H, m); 1.36 (2H, bs); 1.15-0.95 (3H, m). $^{13}\text{C-NMR}$ (CDCl_3): δ =
30 172.9, 158.6, 136.6, 132.7, 129.2, 128.6, 127.9, 127.4, 114.4, 55.5, 55.1, 54.4, 45.2, 45.0, 29.8, 29.2, 21.2, 13.8.

Example 123 - 2-(4-Methoxyphenyl)-N-(1-phenylethyl)-N-(1-methylpiperidin-4-yl) acetamide (47AKU-37)

4-Alpha-methylbenzylamino-1-methyl-piperidine (47AKU-36)

DL-Phenylethylamine (606 mg, 5.0 mmol) was dissolved in 10 ml methanol and 1-Methyl-4-piperidone (566 mg, 5.0 mmol) in 10 ml methanol was added. Mixture was stirred and Acetic acid (~0.75 ml) was added until pH~5. NaCNBH₃ (628 g, 10 mmol) was slowly added. Gas evolution observed. After magnetic stirring for 20 hrs methanol was partly removed on Rotavapor (40°C). Ethylacetate, 2M NaOH and water were added until pH~10. Phases were separated and aq. phase was then re-extracted with ethylacetate and dichloromethane. Combined organic phases were dried over MgSO₄. Concentration on Rotavapor (40°C) yielded 838 mg crude 47AKU-36. TLC (10% methanol in dichloromethane): R_f = 0.3. HPLC-MS (Method A): M⁺ = 219.1 (UV/MS(%)=100/94).

2-(4-Methoxyphenyl)-N-alpha-methylbenzyl-N-(1-methylpiperidin-4-yl) acetamide (47AKU-37)

47AKU-36 (218 mg, 1.0 mmol) was dissolved in 10 ml dichloromethane and placed in 50 ml flask. 4-Methoxyphenylacetyl chloride (185 mg, 1.2 mmol) in 10 ml dichloromethane was added. After 16 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 256 mg (70%) 47AKU-37. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.5. HPLC-MS (Method A): M⁺ = 367.3 (UV/MS(%)=100/99).

¹H-NMR (400 MHz, CDCl₃, rotamers): δ = 7.34-7.06 (7H, m); 6.84 (2H, d); 5.10 (1H, m); 3.77 (3H, s); 3.67 (2H, m); 3.17 (1H, m); 3.03-2.75 (3H, m); 2.64 (3H, s); 2.38 (2H, m); 1.77-1.05 (6H, m). ¹³C-NMR (CDCl₃): δ = 172.0, 158.9, 139.9, 130.0, 129.0, 128.2, 127.1, 114.5, 55.5, 53.1, 51.4, 42.4, 41.3, 31.1, 29.5, 24.9, 18.1.

Example 124 - 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(3-trope-4-yl) acetamide (47AKU-39)

4-Methylbenzylimino-tropane (47AKU-38)

4-Methylbenzylamine (1.21 g, 10 mmol) and Tropinone (1.39 g, 10 mmol) were placed in 100 ml flask and dissolved in 50 ml toluene. Mixture was heated to reflux for 3 hrs and water was removed using a Dean/Stark water-separator. Crude

product was concentrated on Rotavapor (40°C) giving **47AKU-38**. TLC (10% methanol in dichloromethane): $R_f = 0.3$. $^1\text{H-NMR}$ (400 MHz, CDCl_3 , isomers): 7.20-7.09 (4H, m); 4.47 (1H, m); 3.81 (1H, s); 3.42 (1H, m); 3.31 (1H, m); 2.77-2.56 (2H, m); 2.47 and 2.41 (3H, 2s); 2.33 and 2.31 (3H, 2s); 2.27-1.97 (4H, m); 1.69-1.54 (2H, m).

2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(3-tropen-4-yl) acetamide (47AKU-39)

47AKU-38 (242 mg, 1.0 mmol) was dissolved in 5 ml dichloromethane and placed in 50 ml flask. 4-Methoxyphenylacetyl chloride (185 mg, 1.2 mmol) in 10 ml dichloromethane was added. After 16 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 69 mg (18%) **47AKU-39**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): $R_f = 0.4$. HPLC-MS (Method A): $M^+ = 391.2$ (UV/MS(%)=91/86).

$^1\text{H-NMR}$ (400 MHz, CDCl_3 , rotamers): $\delta = 7.22$ -6.82 (8H, m); 5.41 (1H, bs); 4.71-4.52 (2H, m); 3.78 (3H, s); 3.68 (2H, m); 3.44-3.24 (2H, m); 2.72-2.36 (5H, m); 2.32 (3H, s); 2.25-2.00 (2H, m); 1.80-1.54 (2H, m). $^{13}\text{C-NMR}$ (CDCl_3): $\delta = 170.8$, 158.7, 137.4, 134.9, 130.1, 129.3, 128.9, 126.9, 114.2, 59.0, 58.0, 55.5, 49.5, 46.3, 39.7, 35.9, 33.8, 29.7, 21.3.

Example 125 - 2-Phenyl-2-ethyl-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (47AKU-40)

2-Phenylbutyric acid (197 mg, 1.2 mmol) was dissolved in 2 ml thionylchloride and placed in 50 ml flask. Mixture was heated to reflux for 2 hrs and then concentrated on Rotavapor (50°C). The acid chloride (1.2 mmol) in 5 ml dichloromethane was added to **47AKU-5** (158 mg, 0.72 mmol) in 5 ml dichloromethane. After 20 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 196 mg (74%) **47AKU-40**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/

heptane. TLC (10% methanol in dichloromethane): $R_f = 0.5$. HPLC-MS (Method A): $M^+ = 365.4$ (UV/MS(%)=99/100). $^1\text{H-NMR}$ (400 MHz, CDCl_3 , rotamers): $\delta = 7.32$ -6.98 (8H, m); 4.77 (1H, bs); 4.50 (1H, d); 4.29 (1H, d); 3.43 and

3.21 (3H, 2m); 2.72 (2H, m); 2.62 (3H, s); 2.43 (1H, m); 2.32 (3H, s); 2.21 (3H, m); 2.04 (2H, m); 1.67 (3H, m); 0.92-0.72 (3H, m). ^{13}C -NMR (CDCl_3): δ = 174.7, 139.9, 137.3, 135.2, 129.7, 129.0, 127.8, 127.3, 125.8, 54.5, 51.6, 49.4, 46.0, 43.8, 28.9, 26.7, 26.3, 21.2, 12.7.

5 **Example 126 - 2-(4-Methoxyphenyl)-N-(1-indanyl)-N-(1-methylpiperidin-4-yl) acetamide (47AKU-43)**

 4-(1-Indanamino)-1-methyl-piperidine (47AKU-42)

1-Indanamine (666 mg, 5.0 mmol) was dissolved in 10 ml methanol and placed in 100 ml flask. 1-Methyl-4-piperidone (566 mg, 5.0 mmol) in 10 ml methanol
10 was added. Mixture was stirred and Acetic acid (~0.75 ml) was added until pH~5. NaCNBH_3 (628 g, 10 mmol) was slowly added. Gas evolution observed. After magnetic stirring for 16 hrs methanol was partly removed on Rotavapor (40°C). Dichloromethane, 2M NaOH and water were added until pH~10. Phases were separated and aq. phase was then re-extracted with ethylacetate and dichloromethane.
15 Combined organic phases were dried over MgSO_4 . Concentration on Rotavapor (40°C) yielded 1.06 g 47AKU-42. TLC (10% methanol in dichloromethane): R_f = 0.3. HPLC-MS (Method A): M^+ = 231.1 (UV/MS(%)=72/91).

 2-(4-Methoxyphenyl)-N-(1-indanyl)-N-(1-methylpiperidin-4-yl) acetamide (47AKU-43)

20 47AKU-42 (230 mg, 1.0 mmol) was dissolved in 10 ml dichloromethane and placed in 50 ml flask. 4-Methoxyphenylacetyl chloride (185 mg, 1.2 mmol) in 10 ml dichloromethane was added. After 16 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 194 mg (51%)
25 47AKU-43. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.5. HPLC-MS (Method A): M^+ = 379.2 (UV/MS(%)=94/90).

Example 127 - (47AKU-44)N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-(4-methoxybenzyl)-carbamide (47AKU-44)

30 47AKU-5 (219 mg, 1.0 mmol) was dissolved in 5 ml dichloromethane and placed in 50 ml flask. 4-Methoxybenzylisocyanate (196 mg, 1.2 mmol) in 10 ml dichloromethane was added. After 16 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash

chromatography (0-10% methanol in dichloromethane) giving 192 mg (50%)

47AKU-44. HCl-salt was prepared from 2M HCl/diethylether in

dichloromethane/heptane. TLC (10% methanol in dichloromethane): $R_f = 0.3$. HPLC-

MS (Method A): $M^+ = 382.3$ (UV/MS(%)=100/94). $^1\text{H-NMR}$ (400 MHz, CDCl_3): $\delta =$

- 5 7.10 (4H, m); 6.98 (2H, m); 6.76 (2H, m); 4.58 (1H, t); 4.45 (1H, m); 4.33 (2H, s);
4.25 (2H, d); 3.76 (3H, s); 2.97 (2H, m); 2.34 (3H, s); 2.32 (3H, s); 2.24 (2H, m); 1.78
(4H, m). $^{13}\text{C-NMR}$ (CDCl_3): $\delta = 158.9, 158.5, 137.3, 135.2, 131.8, 129.8, 128.8,$
126.2, 114.1, 55.5, 55.4, 51.7, 45.8, 45.7, 44.5, 29.7, 21.2.

Example 128 - 2-(3,4-dimethoxyphenyl)-N-(4-methylbenzyl)-N-(1-
10 **methylpiperidin-4-yl) acetamide (47AKU-45)**

3,4-Dimethoxyphenylbutyric acid (235 mg, 1.2 mmol) was dissolved in 2 ml
thionylchloride and placed in 50 ml flask. Mixture was heated to reflux for 2 hrs and

then concentrated on Rotavapor (50°C). The acid chloride (1.2 mmol) in 5 ml
dichloromethane was added to **47AKU-5** (219 mg, 1.0 mmol) in 10 ml

- 15 dichloromethane. After 16 hrs magnetic stirring the reaction mixture was concentrated
on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10%
methanol in dichloromethane) giving 129 mg (33%) **47AKU-45**. HCl-salt was
prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol
in dichloromethane): $R_f = 0.4$. HPLC-MS (Method A): $M^+ = 397.4$

- 20 (UV/MS(%)=98/89). $^1\text{H-NMR}$ (400 MHz, CDCl_3 , rotamers): $\delta = 7.17-6.60$ (7H, m);
4.75 (1H, m); 4.51 (2H, s); 3.83 (3H, s); 3.79 (3H, s); 3.53 (2H, s); 3.27 (2H, d); 2.65
(2H, t); 2.58 (3H, s); 2.32 (3H, s); 2.24 (2H, m); 1.72 (2H, d). $^{13}\text{C-NMR}$ (CDCl_3): $\delta =$
172.8, 149.3, 148.3, 137.4, 135.0, 129.8, 127.4, 125.8, 121.0, 112.2, 111.6, 56.2, 56.1,
54.6, 49.6, 46.7, 44.0, 40.9, 27.0, 21.2.

25 **Example 129 - 2-(3,4-Methylenedioxyphenyl)-N-(4-methylbenzyl)-N-(1-**
methylpiperidin-4-yl) acetamide (47AKU-46)

3,4-Methylenedioxyphenylacetic acid (216 mg, 1.2 mmol) was dissolved in 2
ml thionylchloride and placed in 50 ml flask. Mixture was heated to reflux for 2 hrs

and then concentrated on Rotavapor (50°C). The acid chloride (1.2 mmol) in 5 ml

- 30 dichloromethane was added to **47AKU-5** (219 mg, 1.0 mmol) in 10 ml
dichloromethane. After 2 hrs magnetic stirring the reaction mixture was concentrated
on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10%
methanol in dichloromethane) giving 188 mg (49%) product. Further purification by

ion exchange chromatography (washout with 10% aq. NH_4OH (25%) in methanol) yielded 149 mg (39%) **47AKU-46**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/

heptane. TLC (10% methanol in dichloromethane): $R_f = 0.4$. HPLC-MS

- 5 (Method A): $M^+ = 381.2$ (UV/MS(%)=96/95). $^1\text{H-NMR}$ (400 MHz, CDCl_3 , rotamers): $\delta = 7.17\text{--}7.02$ (4H, m); 6.77-6.51 (3H, m); 5.91 and 5.93 (2H, 2s); 4.70 (1H, m); 4.52 and 4.49 (2H, 2s); 3.51 (2H, s); 3.26 (2H, d); 2.49 (3H, s); 2.33 (3H, s); 2.14-1.66 (6H, m) $^{13}\text{C-NMR}$ (CDCl_3): $\delta = 172.5, 148.1, 146.8, 137.3, 135.1, 129.8, 128.6, 125.8, 121.9, 109.4, 108.5, 101.2, 54.8, 50.2, 46.7, 44.6, 41.1, 27.7, 21.2$.

10 **Example 130 - 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-t-butylpiperidin-4-yl)-acetamide (47AKU-49)**

1-t-Butyl-4-piperidone (47AKU-47)

- 1-Benzyl-4-piperidone (1.89 g, 10 mmol) was dissolved in 15 ml acetone. Methyl iodide (0.90 ml, 15 mmol) was slowly added over 5 min. After 2 hrs magnetic
15 stirring additional Methyl iodide (1.8 ml, 30 mmol) was added. After 1 hr magnetic stirring 20 ml diethyl-ether was added. Crude product was collected by filtration and washed with acetone/diethylether. White crystals were dried under vacuum giving 806 mg quaternary salt. TLC (10% methanol in dichloromethane): $R_f = 0.7$. Partly dissolved salt in 5 ml water was added to 50°C hot mixture of t-Butylamine (120 mg,
20 1.6 mmol) and Potassiumcarbonate (32 mg, 0.22 mmol) in 3 ml ethanol. The resulting mixture was stirred and heated to reflux (~80°C) for 1 hr. After cooling water (20 ml) and dichloromethane (20 ml) were added. Phases were separated and aq. phase was re-extracted with dichloromethane and ethylacetate. Combined organic phases were dried over MgSO_4 and concentrated on Rotavapor (40°C) giving 496 mg **47AKU-47**.
25 TLC (10% methanol in dichloromethane): $R_f = 0.3$. $^1\text{H-NMR}$ (400MHz, CDCl_3): $\delta = 2.82$ (4H, t); 2.41 (4H, t); 1.12 (9H, s). $^{13}\text{C-NMR}$ (CDCl_3): $\delta = 210.2, 54.3, 46.4, 42.4, 26.6$. Crude product contained ~25% ($^1\text{H-NMR}$) starting material (1-Benzyl-4-piperidone).

4-(4-Methylbenzylamino)-1-t-butyl-piperidine (47AKU-48)

- 30 4-Methylbenzylamine (268 mg, 2.2 mmol) was dissolved in 5 ml methanol and placed in 50 ml flask. **47AKU-47** (305 mg, 2.0 mmol) in 5 ml methanol was added. Acetic acid (0.3 ml) was added until pH~5. NaCNBH_3 (250 mg, 4.0 mmol) was slowly added. Gas evolution observed. After 4 hrs magnetic stirring

dichloromethane, 2M NaOH and water were added until pH~10. Phases were separated and aq. phase was then re-extracted with dichloromethane and ethylacetate. Combined organic phases were dried over MgSO₄. Concentration on Rotavapor (40°C) yielded 556 mg crude **47AKU-48**. TLC (20% methanol in dichloromethane):

5 $R_f = 0.4$. HPLC-MS (Method A): $M^+ = 261.2$ (MS(%)=57).

2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-t-butylpiperidin-4-yl)-acetamide

(47AKU-49)

47AKU-48 (556 mg, 2.1 mmol) was placed in 50 ml flask and 5 ml
10 dichloromethane was added. 4-Methoxyphenylacetyl chloride (739 mg, 4.0 mmol) in 10 ml dichloromethane was added. After 4 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 124 mg (15%) product. Further purification by ion exchange chromatography (washout with 10% aq. NH₄OH
15 (25%) in methanol) gave 91mg (11%) **47AKU-49**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): $R_f = 0.5$. HPLC-MS (Method A): $M^+ = 409.4$
(UV/MS(%)=100/90). ¹H-NMR (400 MHz, CDCl₃): $\delta = 7.11$ (4H, m); 7.03 (2H, d); 6.79 (2H, d); 4.78 (1H, m); 4.56 (2H, s); 3.76 (3H, s); 3.53 (2H, s); 3.43 (2H, m); 2.63
20 (2H, m); 2.47 (2H, m); 2.31 (3H, s); 1.74 (2H, d); 1.36 (9H, s). ¹³C-NMR (CDCl₃): $\delta = 173.0, 158.8, 137.1, 135.3, 129.8, 129.7, 127.0, 125.8, 114.3, 55.6, 55.5, 49.8, 46.5, 46.4, 40.5, 26.7, 25.1, 21.2$.

Example 131 - N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-phenylethyl-carbamide (58AKU-1)

25 **47AKU-5-2** (219 mg, 1.0 mmol) was dissolved in 5 ml dichloromethane and placed in 50 ml flask. Phenethylisocyanate (177 mg, 1.2 mmol) in 5 ml dichloromethane was added. After 6 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-15% methanol in dichloromethane) giving 134 mg (37%)
30 **58AKU-1**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): $R_f = 0.5$. HPLC-MS (Method A): $M^+ = 366.3$ (UV/MS(%)=99/96). ¹H-NMR (400 MHz, CDCl₃): $\delta = 7.21-6.97$ (9H, m); 4.33 (1H, m); 4.26 (1H, m); 4.21 (2H, s); 3.39 (2H, q); 2.85 (2H,

m); 2.67 (2H, t); 2.31 (3H, s); 2.24 (3H, s); 2.06 (2H, m); 1.73-1.57 (4H, m). ¹³C-NMR (CDCl₃): δ= 158.7, 139.5, 137.0, 135.4, 129.7, 128.8, 128.6, 126.3, 126.1, 55.6, 52.2, 46.2, 45.8, 42.2, 36.4, 30.2, 21.2.

Example 132 - *N*-Phenylethyl-*N*-(1-methylpiperidin-4-yl)-*N'*-phenethyl-carbamide (58AKU-2)

4-(2-Phenylethyl)amino-1-methylpiperidine (131 mg, 0.6 mmol) was dissolved in 5 ml dichloromethane and placed in 50 ml flask. Phenethylisocyanate (103 mg, 0.7 mmol) in 5 ml dichloromethane was added. After 4 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (45°C). Crude product was
10 purified by flash chromatography (0-10% methanol in dichloromethane) giving 198 mg (90%) **58AKU-1**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.3. HPLC-MS (Method A): M⁺ = 366.3 (UV/MS(%)=100/100). ¹H-NMR (400 MHz, CDCl₃): δ= 7.33-7.16 (8H, m); 7.01 (2H, m); 4.23 (1H, t); 4.04 (1H, m); 3.47 (2H, q); 3.17 (2H,
15 t); 2.89 (2H, m); 2.78 (2H, t); 2.66 (2H, t); 2.28 (3H, s); 2.05 (2H, m); 1.79-1.59 (4H, m). ¹³C-NMR (CDCl₃): δ= 157.8, 139.6, 139.0, 129.0, 128.9, 128.8, 126.8, 126.7, 55.7, 52.5, 46.2, 44.6, 42.0, 37.3, 36.4, 30.5.

Example 133 - *N*-(4-Methylbenzyl)-*N*-(1-*t*-butylpiperidin-4-yl)-*N'*-(4-methoxybenzyl) carbamide (58AKU-3)

47AKU-5-2 (404 mg, 1.6 mmol) was dissolved in 5 ml dichloromethane and placed in 50 ml flask. 4-Methoxybenzylisocyanate (326 mg, 2.0 mmol) in 5 ml dichloromethane was added. After 20 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (45°C). Crude product was purified three times by flash chromatography (0-20% methanol in dichloromethane and 0-30% methanol in
25 ethylacetate) giving 155 mg (23%) **58AKU-3**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/heptane. TLC (10% methanol in dichloromethane): R_f = 0.3. HPLC-MS (Method A): M⁺ = 424.2 (UV/MS(%)=92/83). ¹H-NMR (400 MHz, CDCl₃): δ= 7.10 (4H, m); 6.99 (2H, m); 6.76 (2H, m); 4.53 (1H, m); 4.35 (3H, s); 4.26 (2H, d); 3.77 (3H, s); 3.09 (2H, m); 2.32 (3H, s); 2.22 (2H, m);
30 1.81-1.54 (4H, m); 1.06 (9H, s). ¹³C-NMR (CDCl₃): δ= 158.9, 158.6, 137.1, 135.6, 131.9, 129.7, 128.8, 126.2, 114.0, 62.6, 55.5, 53.0, 45.9, 45.7, 44.5, 31.0, 26.3, 21.2.

Example 134 - 2-(4-Ethoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (58AKU-4)

4-Ethoxyphenylacetic acid (270 mg, 1.5 mmol) was dissolved in 2 ml thionylchloride and placed in 50 ml flask. Mixture was heated to reflux for 2 hrs and
5 then concentrated on Rotavapor (45°C). The acid chloride (1.5 mmol) in 5 ml dichloromethane was added to 47AKU-5-2 (262 mg, 1.2 mmol) in 5 ml dichloromethane. After 20 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 272 mg (60%) 58AKU-4. HCl-salt was
10 prepared from 2M HCl/diethylether in dichloromethane/

heptane. TLC (10% methanol in dichloromethane): R_f = 0.4. HPLC-MS (Method A): M^+ = 381.2 (UV/MS(%)=98/91). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ = 7.17-6.99 (6H, m); 6.82-6.76 (2H, m); 4.73 (1H, m); 4.48 (2H, s); 3.98 (2H, q); 3.52 (2H, s); 3.22 (2H, d); 2.61 (2H, t); 2.54 (3H, s); 2.32 (3H, s); 2.14 (2H, s); 1.71 (2H, d);
15 1.38 (3H, t). $^{13}\text{C-NMR}$ (CDCl_3): δ = 172.9, 158.2, 137.3, 135.0, 129.9, 129.8, 126.8, 125.8, 114.9, 63.7, 54.6, 49.8, 46.7, 44.1, 40.6, 27.2, 21.2, 15.0.

Example 135 - 2-(4-Butoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide (58AKU-5)

4-Butoxyphenylacetic acid (317 mg, 1.5 mmol) was dissolved in 2 ml
20 thionylchloride and placed in 50 ml flask. Mixture was heated to reflux for 2 hrs and then concentrated on Rotavapor (45°C). The acid chloride (1.5 mmol) in 5 ml dichloromethane was added to 47AKU-5-2 (262 mg, 1.2 mmol) in 5 ml dichloromethane. After 20 hrs magnetic stirring the reaction mixture was concentrated on Rotavapor (40°C). Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) giving 230 mg (47%) 58AKU-5. HCl-salt was
25 prepared from 2M HCl/diethylether in dichloromethane/

heptane. TLC (10% methanol in dichloromethane): R_f = 0.5. HPLC-MS (Method A): M^+ = 409.2 (UV/MS(%)=98/93). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ = 7.15-6.96 (6H, m); 6.78 (2H, m); 4.74 (1H, m); 4.48 (2H, s); 3.91 (2H, t); 3.52 (2H, s);
30 3.27 (2H, d); 2.72 (2H, t); 2.58 (3H, s); 2.32 (3H, s); 2.23 (2H, m); 1.72 (4H, d); 1.45 (2H, m); 0.95 (3H, t). $^{13}\text{C-NMR}$ (CDCl_3): δ = 173.0, 158.4, 137.3, 135.0, 129.8, 126.6, 125.8, 115.0, 67.9, 54.4, 49.5, 46.7, 43.8, 40.6, 31.5, 26.8, 21.2, 19.4, 14.0.

Example 136 - 2-(4-*i*-Propoxyphenyl)-*N*-(4-methylbenzyl)-*N*-(1-methylpiperidin-4-yl) acetamide (58AKU-6)

47AKU-29-2 (245 mg, 0.7 mmol) was dissolved in 10 ml dimethylformamide and placed in 50 ml flask. KOH (196 mg, 3.5 mmol) and Isopropylbromide (200 μ l, 2.1 mmol) were added. Mixture was heated to 50°C and stirred for 24 hrs. After cooling water and ethylacetate were added. Phases were separated and aq. phase was then re-extracted with dichloromethane. Combined organic phases were washed with brine, dried over MgSO₄ and concentrated on Rotavapor (40°C) giving 188 mg. Crude product was purified by flash chromatography (0-10% methanol in dichloromethane) yielding 136 mg (49%) **58AKU-6**. HCl-salt was prepared from 2M HCl/diethylether in dichloromethane/

heptane. TLC (10% methanol in dichloromethane): R_f = 0.3. HPLC-MS (Method B): M⁺ = 395 (UV/MS(%)=95/91). ¹H-NMR (400 MHz, CDCl₃, rotamers): δ = 7.23-7.01 (6H, m); 6.79 (2H, m); 4.60 (1H, m); 4.51 (1H, m); 4.44 (1H, s); 3.77 (1H, s); 3.52 (1H, s); 2.83 (2H, m); 2.76 (2H, m); 2.28 and 2.34 (3H, 2s); 2.19 and 2.22 (3H, 2s); 2.05 (1H, m); 1.86-1.55 (4H, m); 1.32 (6H, d). ¹³C-NMR (CDCl₃): δ = 172.6, 157.0, 137.1, 135.6, 129.8, 129.7, 125.8, 116.2, 70.1, 55.3, 51.6, 46.6, 46.1, 40.8, 29.6, 22.3, 21.2.

Example 137 - Receptor Selection and Amplification (R-SAT) Assays.

The functional receptor assay, Receptor Selection and Amplification Technology (R-SAT), was used (with minor modifications from that previously described US 5,707,798) to screen compounds for efficacy at the 5-HT_{2A} receptor. Briefly, NIH3T3 cells were grown in 96 well tissue culture plates to 70-80% confluence. Cells were transfected for 12-16 hours with plasmid DNAs using superfect (Qiagen Inc.) as per manufacture's protocols. R-SAT's were generally performed with 50 ng/well of receptor and 20 ng/well of Beta-galactosidase plasmid DNA. All receptor and G-protein constructs used were in the pSI mammalian expression vector (Promega Inc) as described in U.S. 5,707,798. The 5HT_{2A} receptor gene was amplified by nested PCR from brain cDNA using the oligodeoxynucleotides based on the published sequence (see Saltzman et. al. *Biochem. Biophys. Res. Comm.* 181:1469-78 (1991)). Large-scale transfections, cells were transfected for 12-16 hours, then trypsinized and frozen in DMSO. Frozen cells were later thawed, plated at 10,000-40,000 cells per well of a 96 well plate that contained drug. With both

methods, cells were then grown in a humidified atmosphere with 5% ambient CO₂ for five days. Media was then removed from the plates and marker gene activity was measured by the addition of the beta-galactosidase substrate ONPG (in PBS with 5% NP-40). The resulting colorimetric reaction was measured in a spectrophotometric plate reader (Titertek Inc.) at 420 nM. All data were analyzed using the computer program XLFit (IDBSm). Efficacy is the percent maximal repression compared to repression by a control compound (ritanserin in the case of 5HT_{2A}). pIC₅₀ is the negative of the log(IC₅₀), where IC₅₀ is the calculated concentration in Molar that produces 50% maximal repression. The results obtained for several compounds of the invention are presented in Table 4, below.

**Table 4. Efficiency and pIC₅₀ of Compounds at the 5-HT_{2A} Receptor
Compared to Ritanserin**

Compound	Percent Efficacy	pIC ₅₀
26HCH17	94	8.3
26HCH65	103	8.2
26HCH66-05	126	8.1
26HCH79-5	94	8.2
26HCH79-6	83	8.3
26HCH79-10	102	7.8
26HCH71B	124	7.9
42ELH45	108	9.0
50ELH27	108	8.7
47AKU-7	120	8.1
42ELH80	122	8.5
42ELH79	110	8.5
42ELH91	108	8.0
42ELH85	118	7.8
42ELH75	109	8.3
47AKU-12	112	8.1
47AKU-8	113	8.1
47AKU-22	117	7.9
47AKU-21	117	7.9
47AKU-20	120	8.0
50ELH8	129	7.8
50ELH68	96	8.4
50ELH65	92	7.9
47AKU-44	112	8.5
57MBT12B	75	7.7
58AKU-4	110	9.6
58AKU-3	111	8.1
58AKU-5	99	9.5
58AKU-6	101	9.8
57MBT54B	95	7.9
50ELH95B	119	8.0
50ELH93E	72	8.1
50ELH93D	58	7.8
50ELH93A	106	8.7
63ELH1A	104	8.3
50ELH89	111	9.7
63ELH20	95	9.0
57MBT70-8D	119	7.7
57MBT70-5D	105	8.4
57MBT70-4D	98	8.5
57MBT70-3D	87	8.9
57MBT70-2D	105	8.2
57MBT70-1D	120	7.9
63ELH21	100	8.5
57MBT62B	119	7.9
57MBT70-6E	115	8.0

Example 138 Selectivity Profile for 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)acetamide hydrochloride

The R-SAT assay (described above in example 137) was used to investigate the selectivity of 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)acetamide hydrochloride. The results from a broad profiling of this compound at a
5 variety of receptors are reported in Table 4 below. NR means No Response, i.e. the compound investigated showed no effect at the receptor studied.

Table 4 – Selectivity of 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)acetamide

RECEPTOR	ASSAY	pEC ₅₀ /pIC ₅₀
5-HT _{1A}	agonist	NR
	antagonist	NR
5-HT _{1B}	agonist	NR
	antagonist	NR
5-HT _{1D}	agonist	NR
	antagonist	NR
5-HT _{1E}	agonist	NR
	antagonist	NR
5-HT _{1F}	agonist	NR
	antagonist	NR
5-HT _{2A}	agonist	NR
	inverse agonist	8.8
5-HT _{2B}	agonist	NR
	inverse agonist	6.9
5-HT _{2C}	agonist	NR
	inverse agonist	7
5-HT ₄	agonist	NR
	inverse agonist	NR
5-HT ₆	agonist	NR
	Inv. Agonist	6.8
5-HT ₇	agonist	NR
	inverse agonist	6.9
m1	agonist	NR
	antagonist	NR
m2	agonist	NR
	antagonist	NR
m3	agonist	NR
	antagonist	NR
m4	agonist	NR
	antagonist	NR
m5	agonist	NR
	antagonist	NR
D1	agonist	NR

	antagonist	NR
D2	agonist	NR
	antagonist	NR
D3	agonist	NR
	antagonist	NR
D5	agonist	NR
	antagonist	NR
Histamine 1	agonist	NR
	Inv. agonist	NR
Histamine 2	agonist	NR
	antagonist	NR
Histamine 3	agonist	NR
	antagonist	NR
alpha-1A(a/c)	agonist	NR
	antagonist	NR
alpha-1B	agonist	NR
	In. Agonist	NR
alpha-2A	agonist	NR
	antagonist	NR
alpha-2B	agonist	NR
	antagonist	NR
alpha-2C	agonist	NR
	antagonist	NR
beta 1	agonist	NR
	antagonist	NR
beta 2	agonist	NR
	antagonist	NR
endothelinB	agonist	NR
CCK-A	agonist	NR
NK-1	agonist	NR
Vasopressin1A	agonist	NR
K-opiod	agonist	NR

Example 139 In Vivo Pharmacology of 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)acetamide hydrochloride(AC-90,179)

Methods

Animals and apparatus

5 Instruments) were used for rat experiments (details on startle apparatus and measures, see Male Non-Swiss Albino mice and male Sprague-Dawley rats (Harlan Sprague-Dawley) were housed (4 mice/cage; 2 rats/cage) in rooms with temperature and humidity controlled and water and food (Harlan Teklad) freely available. Mice were kept on a 12-hr light:dark cycle, whereas rats were kept on a 12-hr reverse
10 light:dark cycle. For locomotor and observation experiments in mice, plastic 20x20x30cm activity cages were equipped with photocell beams (AccuScan Instruments). Startle chambers (San Diego Instruments) were used for rat experiments (for details on startle apparatus and measures, see Mansbach *et al.*, (1988) *Psychopharmacology* 94:507-14).

15

Procedure

Observation for Head Twitches

Mice were treated with 2.5 mg/kg DOI i.p. Five min later, mice were treated with AC-90179 s.c. and placed into activity cages. Ten min later, mice were observed
20 using a repeated sampling technique. Each mouse was observed for 10 sec and rated for presence (1) or absence (0) of head twitch behavior for a total of 6 observations in 15 min and a total head twitch score of 0-6. Each dose combination was tested in a separate group of animals (n=8) and the experimenter was blind to drug conditions. Head twitch scores were averaged followed by analysis of variance (ANOVA) and
25 post-hoc Dunnett's t-test comparisons.

Locomotor Activity

For hyperactivity experiments, mice were treated with 0.3 mg/kg dizocilpine or 3.0 mg/kg d-amphetamine i.p. 15 min before the session. Five minutes after the pretreatment, mice were treated with AC-90179 s.c. and placed into the activity cages.
30 For spontaneous activity, AC-90179 was administered alone. Locomotor data were collected during a 15 min session without habituation in a lit room. Each dose combination was tested in a separate group of animals (n=8). Distance traveled (cm) was calculated and averaged followed by ANOVA and post-hoc Dunnett's t-test comparisons.

Startle Testing

Rats were tested and groups (n=10) matched for levels of startle reactivity and prepulse inhibition (PPI; see Mansbach *et al.*, (1988) *Psychopharmacology* 94:507-14). Two days later, test sessions started and consisted of a 5-min acclimation period with a constant background noise (65 dB), followed by 60 presentations of acoustic stimuli to measure acoustic startle responses. The 60 trials consisted of: twenty two 40-ms presentations of a 120 dB broadband pulse, ten 20-ms presentations of each prepulse intensity (68, 71, 77 dB) 100 ms prior to a 40-msec presentation of a 120 dB broadband pulse, and 8 NOSTIM trials in which no stimuli were delivered in order to assess general motor activation in the rats. Thirty min before testing, rats were treated with sterile water (s.c.), risperidone (1.0 mg/kg, i.p.), or AC-90179 (s.c.). Five min later, rats were administered DOI (0.5 mg/kg, s.c.) or 0.9% saline (s.c.). One-week later, rats were administered the same pretreatment drug or vehicle and crossed over to receive the treatment opposite to that they received the previous week. Startle magnitudes and percent PPI for the three prepulse intensities were calculated as described elsewhere (Bakshi, *et al.*, (1994) *J. Pharmacol. Exp. Ther.* 271:787-94) and ANOVAs with repeated measures performed.

Results

To further characterize the clinical utility of a selective 5-HT_{2A} receptor inverse agonist as a novel antipsychotic agent, AC-90179 was tested in head twitch, locomotor and pre pulse inhibition behavioral models. DOI-treated (2.5 mg/kg, i.p., 15 min) mice exhibited an average head twitch score of 2.6 (\pm 0.3, S.E.M.). AC-90179 (0.1 – 30 mg/kg, s.c., 10 min) caused a dose-related decrease in DOI-induced head-twitches with a minimum effective dose of 1 mg/kg and with higher doses completely eliminating head twitch behavior (Figure 2 A).

In the locomotor experiments (Figure 2 B), mice traveled an average of 794 cm (\pm 122 S.E.M.) after vehicle administration. Dizocilpine (0.3 mg/kg, i.p., 15 min) and *d*-amphetamine (3.0 mg/kg, i.p., 15 min) caused increases in distance traveled with averages of 2625 cm (\pm 312) and 3367 cm (\pm 532), respectively. AC-90179 (0.3 – 10 mg/kg, s.c., 10 min) attenuated the hyperactivity induced by dizocilpine, but not by *d*-amphetamine. The minimum effective dose against dizocilpine was 1 mg/kg, whereas AC-90179 reduced spontaneous locomotor activity only at the highest dose tested (30 mg/kg).

The 3-way repeated measures ANOVA on the PPI data from the AC-90179 groups revealed an overall effect of treatment [$F(1,37) = 27.73$, $p < 0.01$] and a treatment by pretreatment interaction [$F(3,37) = 8.22$, $p < 0.01$] (Figure 2 C). DOI significantly disrupted PPI, and AC-90179 was effective in restoring this disruption especially at the higher doses. AC-90179 did not affect PPI on its own, with no significant effect of pretreatment ($p > 0.05$) on percent PPI. Risperidone was used as a positive control because previous studies in our laboratory have suggested that it is effective in blocking the PPI-disruptive effects of DOI. The 3-way repeated measures ANOVA on the PPI data from the risperidone group also revealed a significant effect of treatment [$F(1,18) = 14.08$, $p < 0.01$] and a treatment by pretreatment interaction [$F(1,18) = 24.48$, $p < 0.01$]. As predicted, risperidone was also effective in restoring PPI in DOI-treated rats. Risperidone also had no effect on PPI by itself, as evidenced by a lack of a pretreatment effect ($p > 0.05$). Since there were no significant interactions with prepulse intensity, the data were collapsed across the three prepulse intensities for graphical purposes.

Since there was a significant pretreatment by treatment interaction, pair-wise 2-way repeated measures ANOVAs were conducted on the saline- and DOI-treated groups. In the vehicle-treated rats, there was no effect of AC-90179 ($p > 0.025$) or risperidone ($p > 0.025$) on PPI. In the DOI-treated groups, there were significant effects of AC-90179 [$F(3,37) = 5.68$, $p < 0.01$] and risperidone [$F(1,18) = 16.73$, $p < 0.01$] on percent PPI.

The 3-way repeated measures ANOVA on startle magnitude from the AC-90179 groups revealed a significant effect of pretreatment [$F(3,37) = 2.89$, $p = 0.048$] and treatment [$F(1,37) = 10.27$, $p < 0.01$] on startle magnitude, but no treatment by pretreatment interaction ($p > 0.05$; Figure 1, panel C inset). Risperidone, on the other hand, had no effect on startle magnitude ($p > 0.05$).

Example 140 – In Vivo Pharmacology of Additional Compounds

The effect of various compounds on head twitch behavior in mice treated with DOI was observed as described above in Example 139. The results are summarized below in Table 5.

The effect of various compounds on head twitch behavior in mice treated with DOI was observed as described in Example 139. Animals received 0.1 -30 mg/kg of the compound indicated via subcutaneous injection. MED indicates the minimum

effective dose at which a statistically significant reduction in head twitching score (described above) was observed. MED = minimum effective dose *in vivo*.

Table 5. Comparison Of Analogs For Their Ability To Attenuate DOI-Induced Head Twitches In Mice.

Compound	MED
26HCH17	30
44ELH45	30
50ELH27	1
42ELH80	< 10
42ELH79	< 10
47AKU-7	< 10
42ELH85	< 10
47AKU-8	< 10
47AKU-12	< 10
47AKU-13	< 10
42ELH91	> 10
42ELH90	~ 10
47AKU-20	< 10
47AKU-19	> 10
47AKU-22	< 10
47AKU-21	> 10
42ELH75	< 10
47AKU-11	~ 10
47AKU-14	< 10
47AKU-18	> 10
50ELH6	< 10
47AKU-33	> 10
47AKU-25	> 10
50ELH65	< 10
50ELH68	< 10
47AKU-49	< 10
47AKU-44	< 10
58AKU-4	< 10
58AKU-5	< 1
50ELH93A	< 10
58AKU-6	< 10
63ELH20	< 10
63ELH21	< 10

5

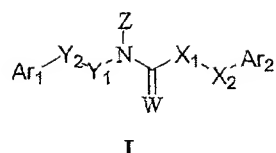
MED = minimum effective dose *in vivo*.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the
5 invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

The disclosures of all references cited herein are incorporated by reference in their entireties.

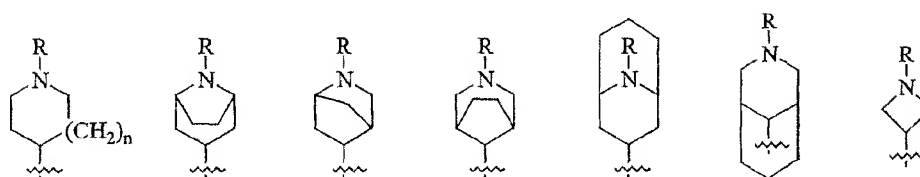
We Claim:

1. A compound of formula (I)



wherein

5 Z is



or

10

in which

R is a hydrogen, a cyclic or straight-chained or branched acyclic organyl group, a lower hydroxyalkyl group, a lower aminoalkyl group, or an aralkyl or heteroaralkyl group;

15 n is 0, 1, or 2;

X₁ is methylene, vinylene, or an NH or N(lower alkyl) group; and

X₂ is methylene, or, when X₁ is methylene or vinylene, X₂ is methylene or a bond; or when X₁ is methylene, X₂ is O, S, NH, or N(lower alkyl) or a bond;

Y₁ is methylene and Y₂ is methylene, vinylene, ethylene, propylene, or a bond;

20 or

Y₁ is a bond and Y₂ is vinylene; or

Y₁ is ethylene and Y₂ is O, S, NH, or N(lower alkyl);

Ar₁ and Ar₂ independently are unsubstituted or substituted aryl or heteroaryl groups, provided that Ar₁ and Ar₂ are not simultaneously phenyl; and

25 W is oxygen or sulfur.

2. A compound according to claim 1, wherein

Y₁ is methylene and Y₂ is a bond, methylene, ethylene, or vinylene; or

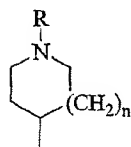
Y_1 is ethylene and Y_2 is O or S;

and

X_1 is methylene and X_2 is a bond, methylene, O, or S; or

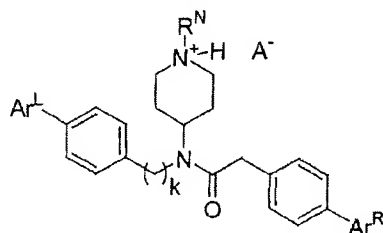
X_1 is NH or N(lower alkyl) and X_2 is methylene.

- 5 3. A compound according to claim 2, wherein
Z is



and W is oxygen.

4. A compound according to claim 3, wherein
- 10 Ar_1 and Ar_2 independently are mono- or disubstituted phenyl groups.
5. A compound according to claim 4, wherein
- R is a hydrogen, a lower alkyl group, a cyclic organyl group, or a substituted or unsubstituted aralkyl or heteroaralkyl group;
- n is 1;
- 15 Y_1 is methylene, Y_2 is a bond, methylene, ethylene, or vinylene;
- X_1 is methylene and X_2 is a bond, or.
- X_1 is NH or N(lower alkyl) and X_2 is methylene; and
- Ar_1 and Ar_2 are phenyl groups, independently *p*-substituted with groups selected from lower alkyl, lower alkoxy and halogen.
- 20 6. A compound according to claim 1, having a formula (II)



II

wherein R^N is hydrogen, lower alkyl, aralkyl, or heteroaralkyl;

Ar^L is selected from lower alkyl, lower alkoxy and halogen

25 Ar^R is selected from lower alkyl, lower alkoxy and halogen;

k is 1 or 2

and A⁻ is a suitable anion.

7. The compound according to claim 1, wherein the compound is selected from the group consisting of:

- N-(1-(1-methylethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- 5 N-(1-(2,2-dimethylethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-pentylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- 10 N-(1-hexylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-cyclohexylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-cyclopentylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- 15 N-(1-cyclobutylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-cyclopropylpiperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- 20 N-(1-(cyclopentylmethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-(cyclobutylmethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-(cyclopropylmethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- 25 N-(1-(2-hydroxyethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- N-(1-(3-hydroxypropyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;
- 30 N-((4-methylphenyl)methyl)-N-(piperidin-4-yl)-N'-phenylmethylcarbamide;
- N-((4-methylphenyl)methyl)-N-(1-(2-methylpropyl)piperidin-4-yl)-N'-phenylmethylcarbamide;
- N-(1-((2-bromophenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide;

N-(1-((4-hydroxy-3-methoxyphenyl)methyl)piperidin-4-yl)-*N*'-((4-methylphenyl)methyl)-*N*'-phenylmethylcarbamide;

N-(1-((5-ethylthien-2-yl)methyl)piperidin-4-yl)-*N*'-((4-methylphenyl)methyl)-*N*'-phenylmethylcarbamide;

5 *N*-(1-(imidazol-2-ylmethyl)piperidin-4-yl)-*N*'-((4-methylphenyl)methyl)-*N*'-phenylmethylcarbamide;

N-(1-(cyclohexylmethyl)piperidin-4-yl)-*N*'-((4-methylphenyl)methyl)-*N*'-phenylmethylcarbamide;

10 *N*-(1-((4-fluorophenyl)methyl)piperidin-4-yl)-*N*'-((4-methylphenyl)methyl)-*N*'-phenylmethylcarbamide;

N'-((4-methylphenyl)methyl)-*N*-(piperidin-4-yl)-4-methoxyphenylacetamide;

N'-((4-methylphenyl)methyl)-*N*-(1-methylpiperidin-4-yl)-4-methoxyphenylacetamide;

15 *N*-(1-ethylpiperidin-4-yl)-*N*'-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;

N'-((4-methylphenyl)methyl)-*N*-(1-propylpiperidin-4-yl)-4-methoxyphenylacetamide;

N-(1-butylpiperidin-4-yl)-*N*'-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;

20 *N*-(1-(3,3-dimethylbutyl)piperidin-4-yl)-*N*'-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;

N-(1-(cyclohexylmethyl)piperidin-4-yl)-*N*'-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;

25 *N*'-((4-methylphenyl)methyl)-*N*-(1-(2-methylpropyl)piperidin-4-yl)-4-methoxyphenylacetamide;

N'-((4-methylphenyl)methyl)-*N*-(1-((4-methylphenyl)methyl)piperidin-4-yl)-4-methoxyphenylacetamide;

N-(1-((4-hydroxyphenyl)methyl)piperidin-4-yl)-*N*'-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;

30 *N*-(1-((2-hydroxyphenyl)methyl)piperidin-4-yl)-*N*'-((4-methylphenyl)methyl)-4-methoxyphenylacetamide;

N-(3-phenylpropyl)-*N*-(piperidin-4-yl)-4-methoxyphenylacetamide;

N-(2-phenylethyl)-*N*-(piperidin-4-yl)-4-methoxyphenylacetamide;

N'-((2-methoxyphenyl)methyl)-*N*-(piperidin-4-yl)-4-methoxyphenylacetamide;

- N-((2-chlorophenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
 N-((3,4-di-methoxyphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
 5 N-((2,4-di-chlorophenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
 N-((3-methylphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
 N-((3-bromophenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide;
 N-(1-(phenylmethyl)piperidin-4-yl)-N-(3-phenyl-2-propen-1-yl)-4-methoxyphenylacetamide;
 10 N-((4-methylphenyl)methyl)-N-(1-piperidin-4-yl)-phenylacetamide;
 N-((4-methylphenyl)methyl)-N-(1-piperidin-4-yl)-3-phenylpropionamide;
 N-((4-methylphenyl)methyl)-N-(1-piperidin-4-yl)-(phenylthio)acetamide;
 N-((4-methylphenyl)methyl)-N-(1-piperidin-4-yl)-phenoxyacetamide;
 15 N-((4-methylphenyl)methyl)-N-(1-piperidin-4-yl)-(4-chlorophenoxy)acetamide;
 N-((4-methylphenyl)methyl)-N-(1-piperidin-4-yl)-3-methoxyphenylacetamide;
 N-((4-methylphenyl)methyl)-N-(1-piperidin-4-yl)-4-fluorophenylacetamide;
 20 N-((4-methylphenyl)methyl)-N-(1-piperidin-4-yl)-2,5-di-methoxyphenylacetamide;
 N-((4-methylphenyl)methyl)-N-(1-piperidin-4-yl)-4-chlorophenylacetamide;
 N-((4-methylphenyl)methyl)-N-(1-(phenylmethyl)pyrrolidin-3-yl)-N'-phenylmethylcarbamide;
 25 N-((4-methylphenyl)methyl)-N-(1-(phenylmethyl)pyrrolidin-3-yl)-4-methoxyphenylacetamide;
 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(piperidin-4-yl) acetamide;
 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide;
 30 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(1-ethylpiperidin-4-yl) acetamide;
 2-(4-methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-ethylpiperidin-4-yl) acetamide.
 2-(4-methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-isopropylpiperidin-4-yl) acetamide;

- 2-(4-methoxyphenyl)-*N*-(4-chlorobenzyl)-*N*-(piperidin-4-yl) acetamide;
2-(4-methoxyphenyl)-*N*-(4-chlorobenzyl)-*N*-(1-cyclopentylpiperidin-4-yl)
acetamide;
2-(4-methoxyphenyl)-*N*-(4-chlorobenzyl)-*N*-(1-isopropylpiperidin-4-yl)
5 acetamide;
2-(phenyl)-*N*-(4-trifluoromethylbenzyl)-*N*-(1-methylpiperidin-4-yl) acetamide;
2-(4-fluorophenyl)-*N*-(4-trifluoromethylbenzyl)-*N*-(1-methylpiperidin-4-yl)
acetamide;
2-(4-Methoxyphenyl)-*N*-(4-trifluoromethylbenzyl)-*N*-(1-methylpiperidin-4-yl)
10 acetamide;
2-(4-Trifluoromethylphenyl)-*N*-(4-trifluoromethylbenzyl)-*N*-(1-
methylpiperidin-4-yl) acetamide;
2-(4-Fluorophenyl)-*N*-(4-fluorobenzyl)-*N*-(1-methylpiperidin-4-yl) acetamide;
2-(4-Methoxyphenyl)-*N*-(4-fluorobenzyl)-*N*-(1-methylpiperidin-4-yl)
15 acetamide;
2-(phenyl)-*N*-(4-fluorobenzyl)-*N*-(1-methylpiperidin-4-yl) acetamide;
2-(4-Trifluoromethylphenyl)-*N*-(4-fluorobenzyl)-*N*-(1-methylpiperidin-4-yl)
acetamide;
2-(4-trifluoromethylphenyl)-*N*-[4-(methoxycarbonyl)benzyl]-*N*-(1-
20 methylpiperidin-4-yl) acetamide;
2-Phenyl-*N*-[4-(methoxycarbonyl)benzyl]-*N*-(1-methylpiperidin-4-yl)
acetamide;
2-(4-Chlorophenyl)-*N*-[4-(methoxycarbonyl)benzyl]-*N*-(1-methylpiperidin-4-
yl) acetamide;
25 2-(4-Methoxyphenyl)-*N*-[4-(methoxycarbonyl)benzyl]-*N*-(1-methylpiperidin-
4-yl) acetamide;
2-(4-trifluoromethylphenyl)-*N*-[4-(methoxycarbonyl)benzyl]-*N*-(1-
methylpiperidin-4-yl) acetamide;
2-Phenyl-*N*-[4-(methoxycarbonyl)benzyl]-*N*-(1-methylpiperidin-4-yl)
30 acetamide;
2-(4-Chlorophenyl)-*N*-[4-(methoxycarbonyl)benzyl]-*N*-(1-methylpiperidin-4-
yl) acetamide;
2-(4-Methoxyphenyl)-*N*-[4-(methoxycarbonyl)benzyl]-*N*-(1-methylpiperidin-
4-yl) acetamide;

2-(4-methoxyphenyl)-*N*-(4-methylbenzyl)-*N*-[1-(4-chloromethyl-2-thiazolylmethyl) piperidin-4-yl] acetamide;

2-(4-methoxyphenyl)-*N*-(4-methylbenzyl)-*N*-{1-[3(1,3-dihydro-2H-benzimidazol-2-on-1-yl) propyl] piperidin-4-yl} acetamide;

5 2-(4-methoxyphenyl)-*N*-(2-(4-fluorophenyl) ethyl)-*N*-(1-methylpiperidin-4-yl) acetamide;

2-(4-methoxyphenyl)-*N*-[2-(2,5-dimethoxyphenyl) ethyl]-*N*-(1-methylpiperidin-4-yl) acetamide;

2-(4-methoxyphenyl)-*N*-[2-(2,4-dichlorophenyl) ethyl]-*N*-(1-methylpiperidin-4-yl) acetamide;

2-(4-methoxyphenyl)-*N*-[2-(3-chlorophenyl) ethyl]-*N*-(1-methylpiperidin-4-yl) acetamide;

2-(4-methoxyphenyl)-*N*-[2-(4-methoxyphenyl) ethyl]-*N*-(1-methylpiperidin-4-yl) acetamide;

15 2-(4-methoxyphenyl)-*N*-[2-(3-fluorophenyl) ethyl]-*N*-(1-methylpiperidin-4-yl) acetamide;

2-(4-ethoxyphenyl)-*N*-[2-(4-fluorophenethyl)-*N*-(1-methylpiperidin-4-yl) acetamide;

2-(4-ethoxyphenyl)-*N*-(4-fluorobenzyl)-*N*-(1-methylpiperidin-4-yl) acetamide;

20 2-(4-methoxyphenyl)-*N*-(4-methylbenzyl)-*N*-{1-[2-(2-hydroxyethoxy)ethyl] piperidin-4-yl} acetamide;

2-(4-methoxyphenyl)-*N*-(4-methylbenzyl)-*N*-[1-((2-chloro-5-thienyl)methyl) piperidin-4-yl] acetamide;

2-(4-methoxyphenyl)-*N*-(4-methylbenzyl)-*N*-[1-(2-(imidazolidinon-1-yl)ethyl)piperidin-4-yl] acetamide;

2-(4-methoxyphenyl)-*N*-(4-methylbenzyl)-*N*-{1-[2-(2,4(1H,3H)quinazolin-3-yl)ethyl] piperidin-4-yl} acetamide;

2-(4-methoxyphenyl)-*N*-(4-methylbenzyl)-*N*-{1-[2-(1,3-dioxolan-2-yl)ethyl]piperidin-4-yl} acetamide;

30 2-(4-methoxyphenyl)-*N*-(4-methylbenzyl)-*N*-{1-[2-(3-indolyl)ethyl] piperidin-4-yl} acetamide;

2-(4-methoxyphenyl)-*N*-(4-methylbenzyl)-*N*-{1-[3-(1,2,4-triazol-1-yl)propyl]piperidin-4-yl} acetamide;

- 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(5-benzofurazanylmethyl)piperidin-4-yl] acetamide;
- 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(5-chlorobenzo[b]thien-3-ylmethyl)piperidin-4-yl] acetamide;
- 5 2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(5-phenyl-1,2,4-oxadiazol-3-ylmethyl)piperidin-4-yl] acetamide;
- 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-isopropylpiperidin-4-yl)-acetamide;
- 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-ethylpiperidin-4-yl)-acetamide;
- 10 2-Phenyl-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide, 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide;
- 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-cyclopentylpiperidin-4-yl)-acetamide;
- 2-(4-Fluorophenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide;
- 15 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-(2-hydroxyethyl)-piperidin-4-yl)-acetamide;
- 2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-cyclobutylpiperidin-4-yl)-acetamide;
- 20 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-cyclobutylpiperidin-4-yl)-acetamide, 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(tropin-4-yl)-acetamide;
- N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-benzyl-carbamide;
- N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-phenyl-carbamide;
- N-Phenethyl-N-(1-methylpiperidin-4-yl)-N'-benzyl-carbamide;
- 25 2-Phenyl-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-acetamide;
- 2-(4-Trifluoromethylphenyl)-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-acetamide;
- 2-(4-Fluorophenyl)-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-acetamide;
- 30 2-(4-Methoxyphenyl)-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-acetamide;
- 2-(4-Methylphenyl)-N-(4-chlorobenzyl)-N-(1-methylpiperidin-4-yl)-acetamide;

- 2-(4-Hydroxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-
acetamide;
N-Phenethyl-N-(1-methylpiperidin-4-yl)-N'-phenyl-carbamide;
N-(3-Phenylpropyl)-N-(1-methylpiperidin-4-yl)-N'-benzyl-carbamide;
5 N-(3-Phenylpropyl)-N-(1-methylpiperidin-4-yl)-N'-phenyl-carbamide;
2-(4-Methoxyphenyl)-2,2-ethylene-N-(4-methylbenzyl)-N-(1-methylpiperidin-
4-yl) acetamide;
2-(4-Methoxyphenyl)-N-alpha-methylbenzyl-N-(1-methylpiperidin-4-yl)
acetamide;
10 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(3-tropen-4-yl) acetamide;
2-Phenyl-2-ethyl-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide;
N-Phenethyl-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-amine;
2-(4-Methoxyphenyl)-N-(1-indanyl)-N-(1-methylpiperidin-4-yl) acetamide;
N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-(4-methoxybenzyl)-
15 carbamide;
2-(3,4-dimethoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)
acetamide;
2-(3,4-Methylenedioxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-
yl) acetamide;
20 2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-t-butylpiperidin-4-yl)-
acetamide;
N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-phenethyl-carbamide;
N-Phenethyl-N-(1-methylpiperidin-4-yl)-N'-phenethyl-carbamide;
N-(4-Methylbenzyl)-N-(1-t-butylpiperidin-4-yl)-N'-(4-methoxybenzyl)-
25 carbamide;
2-(4-Ethoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)
acetamide;
2-(4-Butoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)
acetamide;
30 2-(4-i-Propoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)
acetamide;
2-(4-t-Butoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)
acetamide;

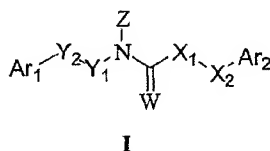
2-(4-Butoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl)
acetamide;

2-(4-Propoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl)
acetamide;

5 2-(4-i-Propoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl)
acetamide; and

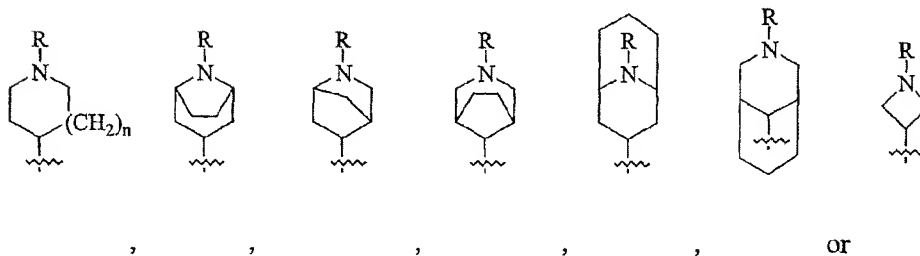
2-(4-t-Butoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl)
acetamide.

8. A compound of formula (I)



10 wherein

Z is



15 in which

R is a hydrogen, a cyclic or straight-chained or branched acyclic organyl
group, a lower hydroxyalkyl group, a lower aminoalkyl group, or an aralkyl or
heteroaralkyl group; and

n is 0, 1, or 2;

20 X₁ is methylene, vinylene, or an NH or N(lower alkyl) group; and

X₂ is methylene, or, when X₁ is methylene or vinylene, X₂ is methylene or a
bond; or when X₁ is methylene, X₂ is O, S, NH, or N(lower alkyl) or a bond;

Y₁ is methylene and Y₂ is methylene, vinylene, ethylene, propylene, or a bond;

or

25 Y₁ is a bond and Y₂ is vinylene; or

Y₁ is ethylene and Y₂ is O, S, NH, or N(lower alkyl);

Ar₁ and Ar₂ are different unsubstituted or substituted aryl or heteroaryl groups;
and

W is oxygen or sulfur.

9. A compound according to claim 8, wherein

5 Y₁ is methylene and Y₂ is a bond, methylene, ethylene, or vinylene; or

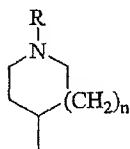
Y₁ is ethylene and Y₂ is O or S; and

X₁ is methylene and X₂ is a bond, methylene, O, or S; or

X₁ is NH or N(lower alkyl) and X₂ is a methylene.

10. A compound according to claim 9, wherein

10 Z is



and W is oxygen.

11. A compound according to claim 10, wherein

15 Ar₁ and Ar₂ independently are mono- or disubstituted phenyl groups.

12. A compound according to claim 11, wherein

R is a hydrogen, a lower alkyl group, a cyclic organyl group, or an, optionally substituted, alalkyl or heteroaralkyl group;

n is 1;

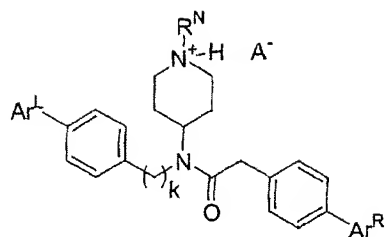
20 Y₁ is methylene, Y₂ is a bond, methylene, ethylene, or vinylene;

X₁ is methylene and X₂ is a bond, or

X₁ is NH or N(lower alkyl) and X₂ is methylene; and

Ar₁ and Ar₂ are phenyl groups, independently p-substituted with groups selected from alkyl, lower alkoxy and halogen.

25 13. A compound according to claim 7, having a formula (II):



II

wherein R^N is hydrogen, lower alkyl, aralkyl, or heteroaralkyl;

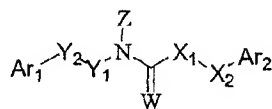
Ar^L is selected from lower alkyl, lower alkoxy and halogen

5 Ar^R is selected from lower alkyl, lower alkoxy and halogen;

k is 1 or 2

and A^- is a suitable anion.

14. A pharmaceutical composition comprising an effective amount of a compound of formula (I):

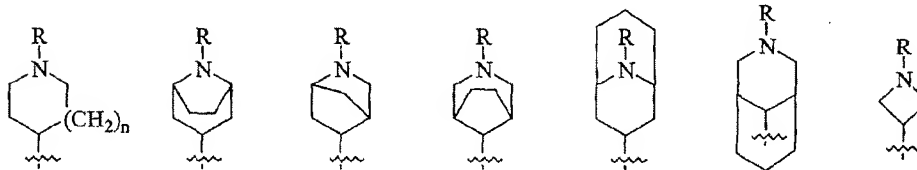


I

10

wherein

Z is



15

in which

R is a hydrogen, a cyclic or straight-chained or branched acyclic organyl group, a lower hydroxyalkyl group, a lower aminoalkyl group, or an aralkyl or heteroaralkyl group; and

20 n is 0, 1, or 2;

X_1 is methylene, vinylene, or an NH or N(lower alkyl) group; and

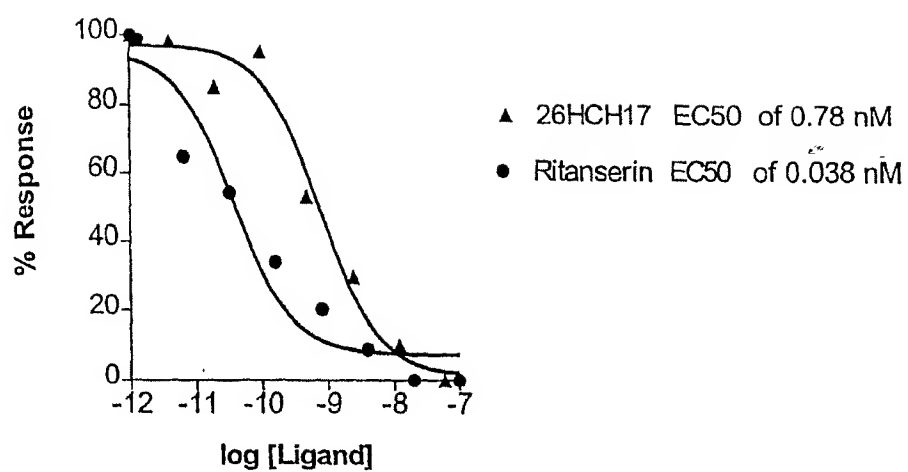
X_2 is methylene, or, when X_1 is methylene or vinylene, X_2 is methylene or a bond; or when X_1 is methylene, X_2 is O, S, NH, or N(lower alkyl) or a bond;

- Y₁ is methylene and Y₂ is methylene, vinylene, ethylene, propylene, or a bond;
or
Y₁ is a bond and Y₂ is vinylene; or
Y₁ is ethylene and Y₂ is O, S, NH, or N(lower alkyl);
5 Ar₁ and Ar₂ independently are unsubstituted or substituted aryl or heteroaryl groups, provided that Ar₁ and Ar₂ are not simultaneously phenyl; and
W is oxygen or sulfur;
or a pharmaceutically acceptable salt, ester or prodrug thereof, and
a pharmaceutically acceptable diluent or excipient.
- 10 15. A method of inhibiting an activity of a monoamine receptor comprising contacting the monoamine receptor or a system containing the monoamine receptor with an amount of one or more of the compounds of claim 1 that is effective in inhibiting the activity of the monoamine receptor.
16. The method of claim 15 wherein the monoamine receptor is a serotonin
15 receptor.
17. The method of claim 16 wherein the serotonin receptor is the 5-HT_{2A} subclass.
18. The method of claim 16 wherein the serotonin receptor is in the central nervous system.
- 20 19. The method of claim 16 wherein the serotonin receptor is in the peripheral nervous system.
20. The method of claim 16 wherein the serotonin receptor is in blood cells or platelets.
21. The method of claim 16 wherein the serotonin receptor is mutated or
25 modified.
22. The method of claim 15 wherein the activity is signaling activity.
23. The method of claim 15 wherein the activity is constitutive.
24. The method of claim 15 wherein the activity is associated with serotonin receptor activation.
- 30 25. A method of inhibiting an activation of a monoamine receptor comprising contacting the monoamine receptor or a system containing the monoamine receptor with an amount of a compound of one or more of the compounds of claim 1 that is effective in inhibiting the activation of the monoamine receptor.
26. The method of claim 25 wherein the activation is by an agonistic agent.

27. The method of claim 26 wherein the agonistic agent is exogenous.
28. The method of claim 26 wherein the agonistic agent is endogenous.
29. The method of claim 25 wherein the activation is constitutive.
30. The method of claim 25 wherein the monoamine receptor is a serotonin
5 receptor.
31. The method of claim 30 wherein the serotonin receptor is the 5-HT2A subclass.
32. The method of claim 30 wherein the serotonin receptor is in the central nervous system.
- 10 33. The method of claim 30 wherein the serotonin receptor is in the peripheral nervous system.
34. The method of claim 30 wherein the serotonin receptor is in blood cells or platelets.
35. The method of claim 30 wherein the serotonin receptor is mutated or
15 modified.
36. A method of treating a disease condition associated with a monoamine receptor comprising administering to a subject in need of such treatment a therapeutically effective amount of one or more of the compounds of claim 1.
37. The method of claim 36 wherein the disease condition is selected from the
20 group consisting of schizophrenia, psychosis, migraine, hypertension, thrombosis, vasospasm, ischemia, depression, anxiety, sleep disorders and appetite disorders.
38. The method of claim 36 wherein the disease condition is associated with dysfunction of a monoamine receptor.
- 25 39. The method of claim 36 wherein the disease condition is associated with activation of a monoamine receptor.
40. The method of claim 36 wherein the disease condition is associated with increased activity of monoamine receptor.
41. The method of claim 36 wherein the monoamine receptor is a serotonin
30 receptor
42. The method of claim 41 wherein the serotonin receptor is the 5-HT2A subclass.
43. The method of claim 41 wherein the serotonin receptor is in the central nervous system.

44. The method of claim 41 wherein the serotonin receptor is in the peripheral nervous system.
45. The method of claim 41 wherein the serotonin receptor is in blood cells or platelets.
- 5 46. The method of claim 41 wherein the serotonin receptor is mutated or modified.
47. A method of treating schizophrenia comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound of one or more of the compounds of claim 1.
- 10 48. A method of treating migraine comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound of one or more of the compounds of claim 1.
49. A method of treating psychosis comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound of one or more of the compounds of claim 1.
- 15 50. A method for identifying a genetic polymorphism predisposing a subject to being responsive to one or more of the compounds of claim 1, comprising: administering to a subject a therapeutically effective amount of the compound; measuring the response of said subject to said compound, thereby identifying a responsive subject having an ameliorated disease condition associated with a monoamine receptor; and
- 20 identifying a genetic polymorphism in the responsive subject, wherein the genetic polymorphism predisposes a subject to being responsive to the compound.
51. The method of claim 50 wherein the ameliorated disease condition is associated with the 5-HT class or 5-HT_{2A} subclass of monoaminergic receptors.
- 25 52. A method for identifying a subject suitable for treatment with one or more of the compounds of claim 1, comprising detecting the presence of a polymorphism in a subject wherein the polymorphism predisposes the subject to being responsive to the compound, and wherein the presence of the polymorphism indicates that the subject is suitable for treatment with one or more of the compounds of claim 1.
- 30

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**FIG. 1**

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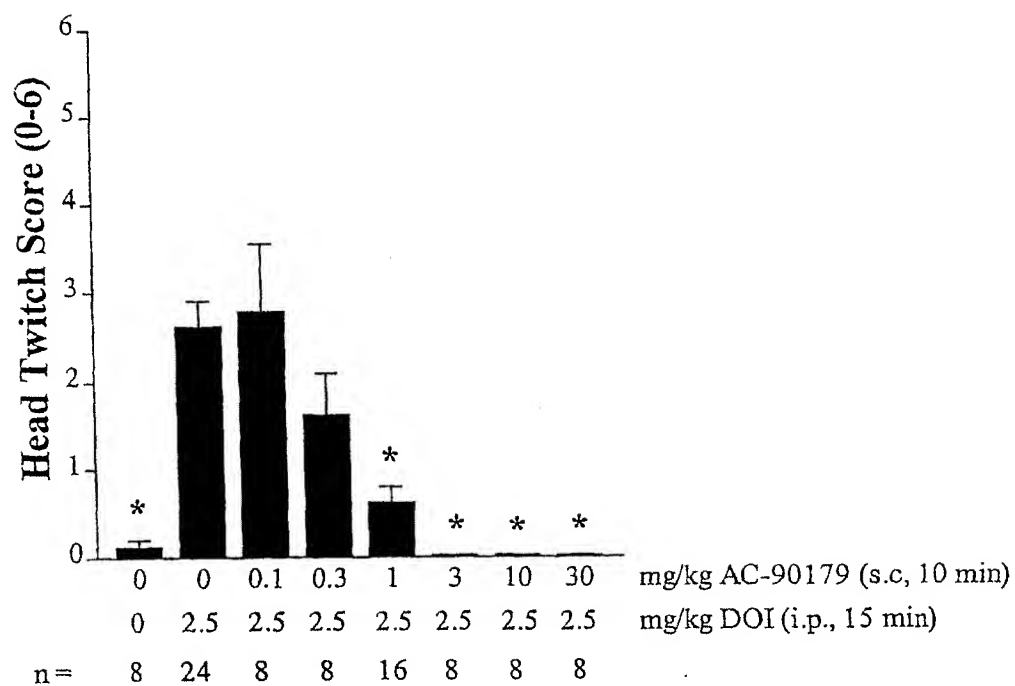
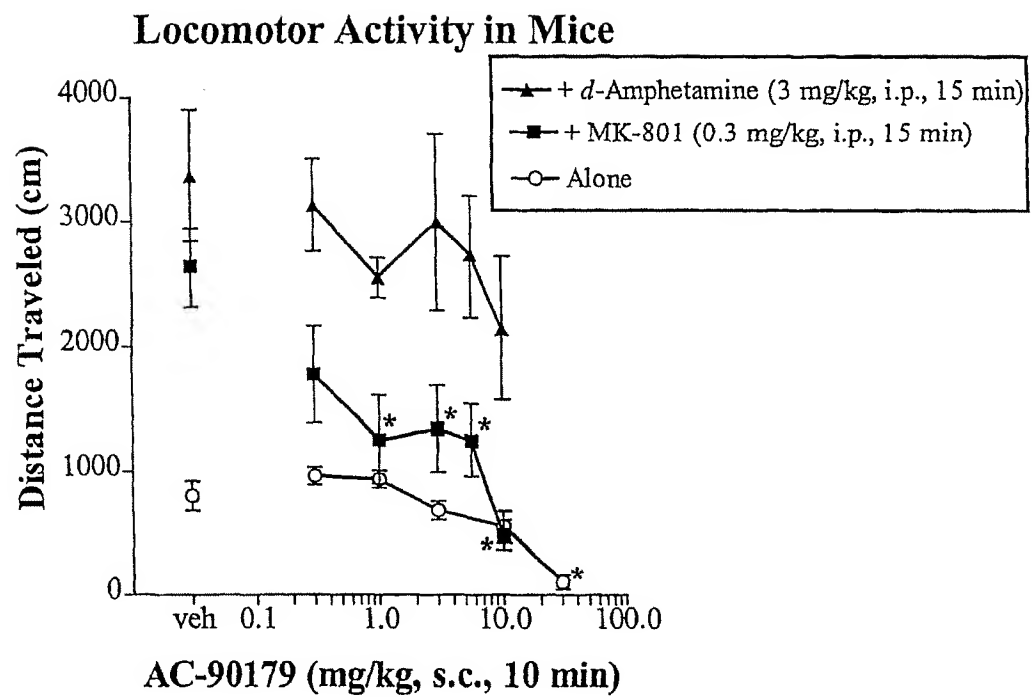


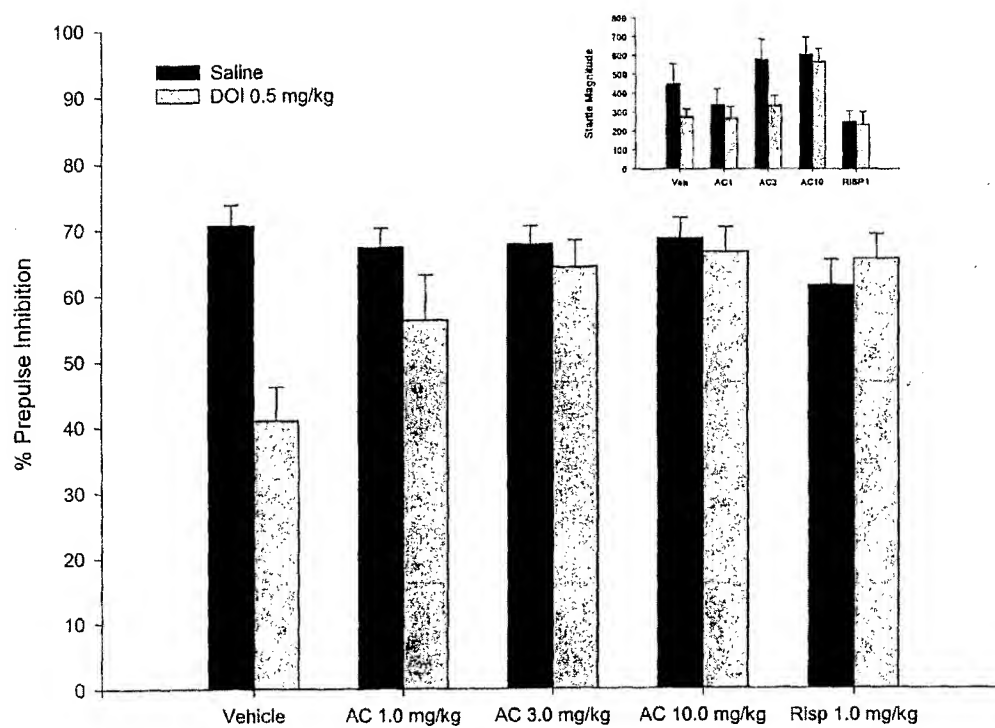
FIG. 2

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**FIG. 3**

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Effects of AC90179 on DOI-induced Disruption of Prepulse Inhibition

**FIG. 4**

INTERNATIONAL SEARCH REPORT

International Application No.

RU/US 01/07187

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07D211/58 C07D207/14 C07D409/06 C07D407/06 C07D401/06
C07D487/08 C07D407/12 A61K31/454 A61P25/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07D A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 853 394 A (KING FRANCIS D ET AL) 1 August 1989 (1989-08-01) abstract examples 1-7 claims 1,3	1,8,14, 15,25, 36, 47-50,52
A	EP 0 260 070 A (LUNDBECK & CO AS H) 16 March 1988 (1988-03-16) the whole document	1,8,14, 15,25, 36, 47-50,52

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

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O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z document member of the same patent family

Date of the actual completion of the international search

4 July 2001

Date of mailing of the international search report

17/07/2001

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Diederer, J

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 01/07187

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4853394 A	01-08-1989	EP 0230718 A JP 62126168 A	05-08-1987 08-06-1987
EP 0260070 A	16-03-1988	AT 92917 T AU 591334 B AU 7823287 A DE 3786966 A DE 3786966 T DK 479587 A,B, ES 2058120 T FI 873924 A,B, IE 60902 B IL 83819 A JP 63077857 A NO 873785 A,B, NZ 221711 A PT 85678 A,B US 4855307 A ZA 8706819 A	15-08-1993 30-11-1989 17-03-1988 16-09-1993 17-03-1994 12-03-1988 01-11-1994 12-03-1988 24-08-1994 16-02-1992 08-04-1988 14-03-1988 28-11-1989 01-10-1987 08-08-1989 29-06-1988

EXHIBIT

7

Exhibit 7

Receptor Selection and Amplification Assay (RSAT™) Protocol to Assess Inverse Agonist Activity in Human 5-HT_{2A} Receptors

Receptor Selection and Amplification (RSAT™) functional assays were carried out essentially as described in *Weiner et al., J. Pharmacol. & Exp. Therap.*, Vol. 299, No.1, pp. 268-79 (2001) (included herewith as **Exhibit 9**) with some modification. NIH-3T3 cells were grown to 70-80% confluence in large vessels in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum and 1% penicillin/streptomycin/L-glutamine (PSG). Cells were transfected with plasmid encoding human 5-HT_{2A} receptor DNA, DNA for G_q G-protein subunit and DNA for beta-galactosidase gene using superfect reagent. After 18-20 hours, the cells were harvested, aliquoted and frozen at -80°C until use. On the day of the assay, the cells were thawed and plated onto 96 well plates containing varying concentration of the ligand under investigation. Cells were incubated in a humidified atmosphere with 5% ambient CO₂ for 5 days. Media were removed from the plates, and beta-galactosidase activity was measured by the addition of *o*-nitrophenyl-D-galactopyranoside in phosphate-buffered saline with 5% Nonidet P-40. The resulting colorimetric reaction was measured in a spectrophotometric plate reader (Titertek, Huntsville, AL) at 420 nm. Data were plotted and analyzed using XLfit software (IDBS, Guildford, Surrey, U.K.).

EXHIBIT

8

Exhibit 8

TABLE I: 5HT_{2A} Receptor Inverse Agonist Activities

Inverse agonist activities are provided as averaged pIEC₅₀ values derived from multiple experimental replicates (n) +/- standard deviation.

Example No. ¹	Chemical Name	Salt Form	5HT _{2A} Inverse Agonist Activity (pIEC ₅₀)
Example A	N-(1-methylpiperidin-4-yl)-N-(4-fluorophenylmethyl)-N'-(4-(2-methylpropyloxy)phenylmethyl)carbamide	Tartrate	9.1 +/- 0.4 (n=16)
Example B	N-(1-methylpiperidin-4-yl)-N-(4-fluorophenylmethyl)-N'-(4-(2-methylpropyloxy)phenylmethyl)carbamide	HCl	9.4 +/- 0.3 (n=22)
Example 1	N-(4-Methylphenyl)methyl)-N-(piperidin-4-yl)-N'-phenylmethylcarbamide	Free Base	7.8 +/- 0.5 (n=10) ²
Example 2	N-(4-Methylphenyl)methyl)-N-(1-(2-methylpropyl)piperidin-4-yl)-N'-phenylmethylcarbamide	Free Base	7.3 +/- 0.6 (n=8)
Example 3	N-(1-(2-Bromophenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide	Free Base	6.7 +/- 0.8 (n=5)
Example 4	N-(1-(4-Hydroxy-3-methoxyphenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide	Free Base	7.0 +/- 0.2 (n=4)
Example 5	N-(1-(5-Ethylthien-2-yl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide	Free Base	7.7 +/- 0.3 (n=3) ³
Example 6	N-(1-(Imidazol-2-yl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide	Free Base	6.6 +/- 0.5 (n=4)
Example 7	N-(1-(Cyclohexylmethyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide	Free Base	8.2 +/- 0.6 (n=9)
Example 8	N-(1-(4-Fluorophenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-N'-phenylmethylcarbamide	Free Base	6.8 +/- 0.5 (n=5)
Example 9	N-(4-methylphenyl)methyl)-N-(1-(phenylmethyl)piperidin-4-yl)-N'-phenylmethylcarbamide	HCl	7.7 +/- 0.7 (n=11) ⁴

¹ Example numbers refer to examples in Andersson.

² Andersson reports an pIEC₅₀ of 8.2.

³ Andersson reports an pIEC₅₀ of 8.1.

⁴ Andersson reports an K_i of 0.9nM.

Example No. ¹	Chemical Name	Salt Form	5HT _{2A} Inverse Agonist Activity (pIEC ₅₀)
Example 10	N-((4-methylphenyl)methyl)-N-(1-(phenylmethyl)piperidin-4-yl)-N'-phenylmethanecarbamide	Free Base	6.9 +/- 0.2 (n=2)
Example 11	N-((4-methylphenyl)methyl)-N-(1-(phenylmethyl)piperidin-4-yl)-4-methoxyphenylacetamide	HCl	8.4 +/- 0.6 (n=24)
Example 13	N-((4-Methylphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	8.0 +/- 0.4 (n=11)
Example 13	N-((4-Methylphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide	HCl	8.0 +/- 0.2 (n=6)
Example 14	N-(1-(3,3-Dimethylbutyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide	Free Base	7.8 +/- 0.5 (n=7) ⁵
Example 15	N-(1-(Cyclohexyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide	Free Base	8.2 +/- 0.4 (n=10)
Example 15	N-(1-(Cyclohexyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide	HCl	9.1 +/- 0.2 (n=7)
Example 16	N-((4-Methylphenyl)methyl)-N-(1-(2-methylpropyl)piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	7.7 +/- 0.4 (n=10)
Example 17	N-(4-Methylphenyl)methyl)-N-(1-(4-methylphenyl)methyl)piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	7.4 +/- 0.4 (n=7)
Example 18	N-(1-(4-Hydroxyphenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide	Free Base	7.6 +/- 0.8 (n=7)
Example 19	N-(1-(2-Hydroxyphenyl)methyl)piperidin-4-yl)-N-((4-methylphenyl)methyl)-4-methoxyphenylacetamide	Free Base	7.5 +/- 0.3 (n=6) ⁶
Example 20	N-(3-Phenylpropyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	7.3 +/- 0.3 (n=6)
Example 21	N-(2-Phenylethyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	7.6 +/- 0.3 (n=8)
Example 22	N-(2-Methoxyphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	NA (n=2)
Example 23	N-(2-Chlorophenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	5.9 +/- 0.2 (n=3)
Example 24	N-(3,4-Dimethoxyphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	NA (n=5)
Example 25	N-(4-Fluorophenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	7.5 +/- 0.4 (n=9)
Example 26	N-(2,4-Dichlorophenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	5.7 (n=1)

⁵ Andersson reports an pIEC₅₀ of 8.2.

⁶ Andersson reports an pIEC₅₀ of 7.8.

Example No. ¹	Chemical Name	Salt Form	5HT _{2A} Inverse Agonist Activity (pIEC ₅₀)
Example 27	N-((3-Methylphenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	6.4 +/- 0.5 (n=6)
Example 28	N-((3-Bromophenyl)methyl)-N-(piperidin-4-yl)-4-methoxyphenylacetamide	Free Base	NA (n=6) ⁷
Example 29	N-(1-(Phenylmethyl)piperidin-4-yl)-N-(3-phenyl-2-propen-1-yl)-4-methoxyphenylacetamide	Free Base	6.7 +/- 0.5 (n=6)
Example 30	N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-phenylacetamide	Free Base	7.3 +/- 0.3 (n=7)
Example 31	N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-3-phenylpropionamide	Free Base	6.6 +/- 0.4 (n=6)
Example 32	N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-(phenylthio)acetamide	Free Base	NA (n=1)
Example 33	N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-phenoxyacetamide	Free Base	6.7 +/- 0.2 (n=2)
Example 34	N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-(4-chlorophenoxy)acetamide	Free Base	8.0 +/- 0.4 (n=3)
Example 35	N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-3-methoxyphenylacetamide	Free Base	7.3 +/- 0.2 (n=6)
Example 36	N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-4-fluorophenylacetamide	Free Base	7.1 +/- 0.1 (n=6)
Example 37	N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-2,5-di-methoxyphenylacetamide	Free Base	NA (n=3)
Example 38	N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-4-chlorophenylacetamide	Free Base	7.8 +/- 0.8 (n=8)
Example 38	N-((4-Methylphenyl)methyl)-N-(1-piperidin-4-yl)-4-chlorophenylacetamide	HCl	7.8 +/- 0.3 (n=6)
Example 39	N-((4-Methylphenyl)methyl)-N-(1-(phenylmethyl)pyrrolidin-3-yl)-N'-phenylmethylcarbamide	Free Base	6.6 +/- 0.1 (n=2)
Example 40	N-((4-Methylphenyl)methyl)-N-(1-(phenylmethyl)pyrrolidin-3-yl)-4-methoxyphenylacetamide	Free Base	6.9 +/- 0.6 (n=4)
Example 46	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	Free Base	NA (n=2)
Example 46	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	8.7 +/- 0.2 (n=16)
Example 48	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(1-ethylpiperidin-4-yl) acetamide	HCl	8.4 +/- 0.4 (n=6)
Example 52	2-(4-methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-cyclopentylpiperidin-4-yl) acetamide	HCl	8.0 +/- 0.2 (n=4)
Example 53	2-(4-methoxyphenyl)-N-(4-chlorobenzyl)-N-(1-isopropylpiperidin-4-yl) acetamide	HCl	7.4 +/- 0.2 (n=6)
Example 54	2-(phenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	6.4 +/- 0.0 (n=2)

⁷ Andersson reports an pIEC₅₀ of 7.21.

Example No. ¹	Chemical Name	Salt Form	5HT _{2A} Inverse Agonist Activity (pIEC ₅₀)
Example 55	2-(4-fluorophenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	6.1 +/- 0.2 (n=2)
Example 56	2-(4-Methoxyphenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.7 +/- 0.0 (n=2)
Example 57	2-(4-Trifluoromethylphenyl)-N-(4-trifluoromethylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	6.5 +/- 0.0 (n=2)
Example 58	2-(4-Fluorophenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.2 +/- 0.2 (n=4)
Example 59	2-(4-Methoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.8 +/- 0.2 (n=7)
Example 60	2-(phenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.5 +/- 0.1 (n=5)
Example 61	2-(4-Trifluoromethylphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.5 +/- 0.2 (n=4)
Example 63	2-(4-trifluoromethylphenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide	HCl	NA (n=2)
Example 64	2-Phenyl-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide	HCl	NA (n=2)
Example 66	2-(4-Methoxyphenyl)-N-[4-(methoxycarbonyl)benzyl]-N-(1-methylpiperidin-4-yl) acetamide	HCl	NA (n=5)
Example 68	2-(4-methoxyphenyl)-N-(3-phenyl-1-propyl)-N-(1-methylpiperidin-4-yl) acetamide	TFA	7.6 +/- 0.4 (n=4)
Example 68	2-(4-methoxyphenyl)-N-(3-phenyl-1-propyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.9 +/- 0.2 (n=2)
Example 69	2-(4-methoxyphenyl)-N-[2-(4-methylphenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide	TFA	8.3 +/- 0.1 (n=2)
Example 69	2-(4-methoxyphenyl)-N-[2-(4-methylphenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide	HCl	8.7 +/- 0.4 (n=4)
Example 70	2-(4-methoxyphenyl)-N-[2-(2-thienyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.5 +/- 0.1 (n=2)
Example 71	2-(4-methoxyphenyl)-N-[2-(4-nitrophenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.5 +/- 0.2 (n=3)
Example 72	2-(4-Methoxyphenyl)-N-(2-thienylmethyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.3 (n=1)
Example 73	2-(4-Methoxyphenyl)-N-(furfuryl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	NA (n=4)
Example 74	2-(2-thienyl)-N-(4-methylphenylmethyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.7 +/- 0.3 (n=5)
Example 75	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-(1-cyclopentylpiperidin-4-yl) acetamide	HCl	8.3 +/- 0.4 (n=6)
Example 76	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[3(1,3 dihydro-2H-benzimidazol-2-on-1-yl) propyl] piperidin-4-yl} acetamide	TFA	9.6 +/- 0.2 (n=6)

Example No. ¹	Chemical Name	Salt Form	5HT _{2A} Inverse Agonist Activity (pIEC ₅₀)
Example 76	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[3(1,3 dihydro-2H-benzimidazol-2-on-1-yl) propyl] piperidin-4-yl} acetamide	HCl	9.3 +/- 0.1 (n=3)
Example 77	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(2-methylthiazol-4-yl)methyl] piperidin-4-yl] acetamide	HCl	8.3 +/- 0.2 (n=3)
Example 78	2-(4-methoxyphenyl)-N-(2-(4-fluorophenyl) ethyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	8.7 +/- 0.3 (n=7)
Example 79	2-(4-methoxyphenyl)-N-[2-(2,5-dimethoxyphenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide	TFA	NA (n=5)
Example 80	2-(4-methoxyphenyl)-N-[2-(2,4-dichlorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide	TFA	7.6 +/- 0.3 (n=3)
Example 80	2-(4-methoxyphenyl)-N-[2-(2,4-dichlorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide	Tartrate	6.4 (n=1)
Example 81	2-(4-methoxyphenyl)-N-[2-(3-chlorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide	TFA	7.8 +/- 0.2 (n=3)
Example 82	2-(4-methoxyphenyl)-N-[2-(4-methoxyphenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide	TFA	7.4 +/- 0.4 (n=3) ⁸
Example 83	2-(4-methoxyphenyl)-N-[2-(3-fluorophenyl) ethyl]-N-(1-methylpiperidin-4-yl) acetamide	TFA	7.9 +/- 0.4 (n=3)
Example 84	2-(4-ethoxyphenyl)-N-[2-(4-fluorophenyl)ethyl]-N-(1-methylpiperidin-4-yl) acetamide	HCl	8.8 +/- 0.2 (n=9) ⁹
Example 85	2-(4-ethoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	8.7 +/- 0.4 (n=8)
Example 86	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(3-hydroxy-4-methoxyphenyl) acetamide	Free Base	7.7 +/- 0.0 (n=1)
Example 87	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(3,4-dihydroxyphenyl) acetamide	Free Base	NA (n=2)
Example 88	N-((3-hydroxy-4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-methoxyphenyl) acetamide	Free Base	7.9 +/- 0.1 (n=2)
Example 89	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-bromophenyl) acetamide	HCl	8.2 +/- 0.3 (n=5)
Example 90	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-iodophenyl) acetamide	HCl	8.4 +/- 0.3 (n=4)
Example 91	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-(2-propyl)phenyl) acetamide	HCl	9.1 +/- 0.3 (n=7)
Example 92	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-trifluoromethoxyphenyl) acetamide	HCl	8.6 +/- 0.3 (n=3)

⁸ Andersson reports an pIEC₅₀ of 8.0.

⁹ Andersson reports an pIEC₅₀ of 9.0.

Example No. ¹	Chemical Name	Salt Form	5HT _{2A} Inverse Agonist Activity (pIEC ₅₀)
Example 93	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-methylthiophenyl) acetamide	HCl	8.5 +/- 0.2 (n=3)
Example 94	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-(N,N'-dimethylamino)phenyl) acetamide	HCl	8.2 +/- 0.4 (n=3)
Example 95	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-nitrophenyl) acetamide	HCl	7.2 +/- 0.0 (n=3)
Example 96	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-methoxy-3-methylphenyl) acetamide	HCl	7.7 +/- 0.0 (n=2)
Example 97	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-pyridyl) acetamide	di-HCl	NA (n=4)
Example 98	N-((4-methylphenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-methylphenyl) acetamide	HCl	8.0 +/- 0.2 (n=4)
Example 99	N-((4-(hydroxymethyl)phenyl)methyl)-N-(1-methylpiperidin-4-yl)-2-(4-methoxyphenyl) acetamide	HCl	6.9 +/- 0.2 (n=2)
Example 100	2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide	HCl	8.0 +/- 0.2 (n=4)
Example 101	2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-ethylpiperidin-4-yl)-acetamide	HCl	8.1 +/- 0.1 (n=4)
Example 102	2-Phenyl-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide	HCl	7.5 +/- 0.3 (n=4)
Example 103	2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide	HCl	8.1 +/- 0.2 (n=4)
Example 104	2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-cyclopentylpiperidin-4-yl)-acetamide	HCl	7.1 +/- 0.0 (n=1)
Example 105	2-(4-Fluorophenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide	HCl	7.5 +/- 0.5 (n=5)
Example 106	2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-(2-hydroxyethyl)-piperidin-4-yl)-acetamide	HCl	6.9 +/- 0.0 (n=2)
Example 107	2-(4-Chlorophenyl)-N-(4-methylbenzyl)-N-(1-cyclobutylpiperidin-4-yl)-acetamide	Oxalate	6.7 +/- 0.0 (n=2)
Example 108	2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-cyclobutylpiperidin-4-yl)-acetamide	Oxalate	8.0 +/- 0.5 (n=4)
Example 109	2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(tropin-4-yl)-acetamide	Oxalate	7.9 +/- 0.4 (n=4)
Example 110	N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-benzyl-carbamide	Oxalate	7.9 +/- 0.2 (n=5)
Example 111	N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-phenyl-carbamide	HCl	NA (n=3)
Example 112	N-Phenethyl-N-(1-methylpiperidin-4-yl)-N'-benzyl-carbamide	HCl	7.4 +/- 0.1 (n=4)
Example 113	2-Phenyl-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-acetamide	HCl	6.5 +/- 0.0 (n=2)
Example 114	2-(4-Trifluoromethylphenyl)-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-acetamide	HCl	6.8 +/- 0.2 (n=2)

Example No. ¹	Chemical Name	Salt Form	5HT _{2A} Inverse Agonist Activity (pIEC ₅₀)
Example 115	2-(4-Fluorophenyl)-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-acetamide	HCl	6.3 +/- 0.1 (n=2)
Example 116	2-(4-Methoxyphenyl)-N-(4-methoxybenzyl)-N-(1-methylpiperidin-4-yl)-acetamide	HCl	7.2 +/- 0.1 (n=2)
Example 117	2-(4-Methylphenyl)-N-(4-chlorobenzyl)-N-(1-methylpiperidin-4-yl)-acetamide	HCl	7.1 +/- 0.1 (n=4)
Example 118	2-(4-Hydroxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-acetamide	HCl	7.1 +/- 0.2 (n=3)
Example 119	N-Phenethyl-N-(1-methylpiperidin-4-yl)-N'-phenyl-carbamide	HCl	NA (n=4)
Example 120	N-(3-Phenylpropyl)-N-(1-methylpiperidin-4-yl)-N'-benzyl-carbamide	HCl	7.1 +/- 0.4 (n=3)
Example 121	N-(3-Phenylpropyl)-N-(1-methylpiperidin-4-yl)-N'-phenyl-carbamide	HCl	6.2 +/- 0.5 (n=4)
Example 122	2-(4-Methoxyphenyl)-2,2-ethylene-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.2 +/- 0.1 (n=2)
Example 123	2-(4-Methoxyphenyl)-N-alpha-methylbenzyl-N-(1-methylpiperidin-4-yl) acetamide	HCl	NA (n=6)
Example 124	2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(3-tropan-4-yl) acetamide	HCl	NA (n=4)
Example 125	2-Phenyl-2-ethyl-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	7.4 +/- 0.2 (n=2)
Example 126	2-(4-Methoxyphenyl)-N-(1-indanyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	NA (n=2)
Example 127	N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-(4-methoxybenzyl)-carbamide	HCl	8.5 +/- 0.3 (n=4)
Example 128	2-(3,4-dimethoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	<7 (n=2)
Example 129	2-(3,4-Methylenedioxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	<7.0 (n=2)
Example 130	2-(4-Methoxyphenyl)-N-(4-methylbenzyl)-N-(1-t-butylpiperidin-4-yl)-acetamide	HCl	7.5 +/- 0.0 (n=2)
Example 131	N-(4-Methylbenzyl)-N-(1-methylpiperidin-4-yl)-N'-phenylethylcarbamide	HCl	7.4 +/- 0.4 (n=2)
Example 132	N-Phenylethyl-N-(1-methylpiperidin-4-yl)-N'-phenylethylcarbamide	HCl	7.1 +/- 0.4 (n=4)
Example 133	N-(4-Methylbenzyl)-N-(1-t-butylpiperidin-4-yl)-N'-(4-methoxybenzyl)-carbamide	HCl	8.1 +/- 0.1 (n=3)
Example 134	2-(4-Ethoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	9.3 +/- 0.3 (n=7) ¹⁰
Example 135	2-(4-Butoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	9.5 +/- 0.2 (n=6)

¹⁰ Andersson reports an pIEC₅₀ of 9.6.

Example No. ¹	Chemical Name	Salt Form	5HT _{2A} Inverse Agonist Activity (pIEC ₅₀)
Example 136	2-(4-i-Propoxyphenyl)-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl) acetamide	HCl	9.7 +/- 0.1 (n=7)
Example P-1 ¹¹	2-(4-Butoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide (Andersson, page 27, line 23)	HCl	9.1 +/- 0.5 (n=6)
Example P-2	2-(4-Propoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide (Andersson, page 27, line 25)	HCl	10.4 +/- 0.2 (n=8)
Example P-3	2-(4-i-Propoxyphenyl)-N-(4-fluorobenzyl)-N-(1-methylpiperidin-4-yl) acetamide (Andersson, page 27, line 27)	HCl	8.8 +/- 0.3 (n=5)
Example P-4	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[2-(2-hydroxyethoxy)ethyl]piperidin-4-yl} acetamide (Andersson, page 25, line 10)	HCl	7.5 +/- 0.0 (n=2)
Example P-5	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-((2-chloro-5-thienyl)methyl)piperidin-4-yl] acetamide (Andersson, page 25, line 12)	HCl	7.2 (n=1)
Example P-6	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(2-imidazolidonon-1-yl)ethyl]piperidin-4-yl] acetamide (Andersson, page 25, line 14)	HCl	8.1 +/- 0.2 (n=3)
Example P-7	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[2-(2,4-(1H, 3H)quinazolin-3-yl)ethyl]piperidin-4-yl} acetamide (Andersson, page 25, line 16)	HCl	7.7 +/- 0.2 (n=2)
Example P-8	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[2-(1,3-dioxolan-2-yl)ethyl]piperidin-4-yl} acetamide (Andersson, page 25, line 18)	HCl	8.5 +/- 0.2 (n=3)
Example P-9	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-{1-[2-(3-indolyl)ethyl]piperidin-4-yl} acetamide (Andersson, page 25, line 20)	HCl	8.3 +/- 0.2 (n=4)
Example P-10	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(5-chlorobenzo[b]thien-3-yl)methyl]piperidin-4-yl] acetamide (Andersson, page 25, line 26)	HCl	7.3 (n=1)
Example P-11	2-(4-methoxyphenyl)-N-(4-methylbenzyl)-N-[1-(5-phenyl-1,2,4-oxadiazol-3-yl)methyl]piperidin-4-yl] acetamide (Andersson, page 25, line 28)	HCl	7.3 +/- 0.1 (n=2)
Example P-12	N-Phenethyl-N-(4-methylbenzyl)-N-(1-methylpiperidin-4-yl)-amine (Andersson, page 27, line 1)	HCl	NA (n=2)

¹¹ Refers to compounds recited in Andersson at pages 25-27.

EXHIBIT

9

5-Hydroxytryptamine_{2A} Receptor Inverse Agonists as Antipsychotics

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ABSTRACT

We have used a cell-based functional assay to define the pharmacological profiles of a wide range of central nervous system active compounds as agonists, competitive antagonists, and inverse agonists at almost all known monoaminergic G-protein-coupled receptor (GPCR) subtypes. Detailed profiling of 40 antipsychotics confirmed that as expected, most of these agents are potent competitive antagonists of the dopamine D₂ receptor. Surprisingly, this analysis also revealed that most are potent and fully efficacious 5-hydroxytryptamine (5-HT)_{2A} receptor inverse agonists. No other molecular property was shared as universally by this class of compounds. Furthermore, comparisons of receptor potencies revealed that antipsychotics with the highest extrapyramidal side effects (EPS)

liability are significantly more potent at D₂ receptors, the EPS-sparing atypical agents had relatively higher potencies at 5-HT_{2A} receptors, while three were significantly more potent at 5-HT_{2A} receptors. Functional high-throughput screening of a diverse chemical library identified 530 ligands with inverse agonist activity at 5-HT_{2A} receptors, including several series of compounds related to known antipsychotics, as well as a number of novel chemistries. An analog of one of the novel chemical series, AC-90179, was pharmacologically profiled against the remaining monoaminergic GPCRs and found to be a highly selective 5-HT_{2A} receptor inverse agonist. The behavioral pharmacology of AC-90179 is characteristic of an atypical antipsychotic agent.

In the past, ligand-binding methodologies have revealed that competitive antagonism of dopamine D₂ receptors is a shared property of most antipsychotic drugs (Creese et al., 1976; Seeman et al., 1976). However, antipsychotics also interact with many additional monoaminergic GPCRs at clinically relevant concentrations, and these additional interactions likely contribute to the differences in the clinical profiles of these agents. The completion of the human genome project, with an estimate of nearly 600 GPCR genes, has increased the number of potential receptor interactions for existing drugs (International Human Genome Consortium, 2001), yet the functional nature and extent of these interactions have not yet been explored because of the limitations of existing methodologies. Current antipsychotic drugs have limited efficacy in many patients and possess debilitating

side effects. While recent efforts have produced newer and improved antipsychotics (Meltzer and McGurk, 1999), these "atypical" agents also have broad ranges of molecular actions (Bymaster et al., 1996) and possess unique side effect profiles that were not exhibited by older agents. Therefore, an improved understanding of the functional molecular profiles of existing drugs, including antipsychotics, may identify unique drug targets to exploit or to expressly avoid.

Previous attempts to determine the molecular profiles of antipsychotic drugs have utilized ligand-binding techniques to characterize drug actions at various receptors. These studies have revealed a tremendous degree of heterogeneity in potential sites of drug/receptor interactions and have highlighted the lack of selectivity of most antipsychotic agents in routine clinical use. However, these methodologies often could not provide accurate information on agonist affinities at these receptors, nor could these techniques appreciate subtleties in agonist-independent signaling. Indeed, many drugs previously thought to be competitive antagonists actually have intrinsic activity as inverse agonists at monoamin-

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ABBREVIATIONS: GPCR, G-protein coupled receptor; 5-HT, 5-hydroxytryptamine; DMSO, dimethyl sulfoxide; m5CAM, m5 constitutively activated mutant; R-SAT, Receptor Selection and Amplification; DMEM, Dulbecco's modified essential media; PSG, penicillin/streptomycin/glutamine; DOI, (+)-2,5-dimethoxy-4-iodoamphetamine hydrochloride; ANOVA, analysis of variance; PPI, prepulse inhibition.

ergic receptors and can attenuate the basal, constitutive activity of these receptors (Leftkowitz et al., 1993; Milligan et al., 1995; Spalding et al., 1995). The elucidation of the physiological role of these various receptor subtypes, and the clinical relevance of inverse agonism versus competitive antagonism, are among the most relevant of current challenges in pharmacology.

Using a cell-based functional assay, we have generated detailed pharmacologic profiles of therapeutically relevant compounds against most monoaminergic GPCRs. We report the finding that nearly all clinically useful antipsychotics are potent, fully efficacious, inverse agonists at the 5-HT_{2A} receptor, in addition to their known activity as potent dopamine D₂ receptor antagonists. Based on this finding, we launched a drug screening and chemical-lead optimization effort that has identified novel, potent, and selective 5-HT_{2A} inverse agonists. We show that one of these novel 5-HT_{2A} inverse agonists displays a behavioral pharmacological profile similar to that of existing atypical antipsychotic agents.

Materials and Methods

Molecular Cloning

Cloning of the known human monoamine GPCRs was performed using PCR. In brief, oligonucleotides flanking the coding region of each receptor were synthesized based on sequences deposited to GenBank. General PCR conditions used 100 ng (~125 pmol) of each primer, 250 μ M deoxynucleoside-5'-triphosphates, 5% DMSO, 80 ng of genomic DNA or 25 ng of cDNA, 1 \times *Pfu* cloned buffer and 2.5 units of *Pfu* Turbo (Stratagene, San Diego, CA). The standard cycling conditions were as follows: 94 to 98°C for 5 min then 40 cycles of 94 to 98°C for 15 s, 45 to 65°C for 10 s, and 72°C for 1 min/kilobase. The clonings of the dopamine D₁ receptor (Sunahara et al., 1990), D₂ receptor short isoform (Stormann et al., 1990), D₄ receptor (MacKenzie et al., 1994), D₅ receptor (Sunahara et al., 1991), the m1 to m5 muscarinic receptors (Bonner et al., 1987, 1988), and the m5 constitutively activated mutant (m5CAM) (Spalding et al., 1995) were reported previously. The α subunit of Gq, and the G-protein chimera Gqi5, were gifts from Dr. B. Conklin (Gladstone Institute, University of California at San Francisco, San Francisco, CA). All receptor and G-protein constructs were sequence verified.

Receptor Selection and Amplification (R-SAT) Assays

R-SAT (ACADIA Pharmaceuticals, San Diego, CA) assays were performed with minor modifications from that previously described (Spalding et al., 1995; Brauner-Osborne and Brann, 1996; Burstein et al., 1997a,b). Briefly, NIH-3T3 cells were grown in 96-well tissue culture plates to 70 to 80% confluence in Dulbecco's modified essential media (DMEM) supplemented with 10% calf serum and 1% penicillin/streptomycin/glutamine (PSG). Cells were transfected for 12 to 16 h with plasmid DNAs using Superfect Reagent (Qiagen, Valencia, CA) per manufacturer's protocols. R-SATs were generally performed with 1 to 50 ng/well receptor and 20 ng/well β -galactosidase plasmid DNA. For G-protein coexpression studies, 4 to 20 ng/well Gq or Gqi5 was used. After overnight transfection, medium was replaced with serum-free DMEM containing 2% cyto-sf3 (Kemp Biotechnologies, Frederick, MD), and 1% PSG and varying concentrations of drug. Cells were grown in a humidified atmosphere with 5% ambient CO₂ for 4 to 6 days. Medium was removed from the plates, and β -galactosidase activity was measured by the addition of *o*-nitrophenyl β -D-galactopyranoside (in phosphate-buffered saline with 5% Nonidet P-40 detergent). The resulting colorimetric reaction was measured in a spectrophotometric plate reader (Titertek, Huntsville, AL) at 420 nm. All data were analyzed using the computer programs Excel Fit and GraphPad Prism software (San Diego, CA).

Drugs

All compounds for R-SAT studies were solubilized as 10 mM stock solutions in either water or DMSO. Working dilutions were made from 50 μ M solutions in DMEM with 2% cyto-sf3, 1% PSG. All compounds were obtained from Sigma/RBI (Natick, MA) except as follows: spiperone and remoxipride (Tocris, St. Louis, MO) tiospirone (Bristol Myers Squibb, Stamford, CT), amperozide (Upjohn), chlorprothizene (Orgasynth Industries, Glasse, France), prothipendyl (Asta Medica), sultopride (IC Rom, Milan, Italy), moperone and bromperidol (Janssen Research Foundation, Beerse, Belgium), perazine (Byk Gulden, Singen, Germany), sertindole, *trans*-flupenthixol, and molindone (Lundbeck A/S, Copenhagen, Denmark), mesoridazine (BIOMOL Research Laboratories, Plymouth Meeting, PA), melperone (Cilag, Schaffhausen, Switzerland), while AC-90179 and M100907 were synthesized by ACADIA Pharmaceuticals. For behavioral studies, AC-90179 was dissolved in 10% Tween 80 (90% water), dizocilpine (MK-801) and *d*-amphetamine sulfate were dissolved in 0.9% saline, and (+)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) was dissolved in water. All compounds were administered in a volume of 0.1 ml/10 g of body weight, and doses were calculated based on the weight of the salt.

Compound Library and Screening

The compound library screened consisted of 130,000 diverse small organic molecules with drug-like properties. Compounds were stored at -20°C in 100% DMSO, diluted to 30 μ M in water, adding 10% of the final volume to the assay plates for a 3 μ M screening concentration. Coexpression of Gq was used to augment both m5 and 5-HT_{2A} receptor constitutive activity. After transient transfection in roller bottles, cells were trypsinized, harvested, and frozen. Cells were thawed rapidly in DMEM media contained 0.5% calf serum and 2% cyto-sf3 and then added to 96- or 384-well microtiter plates containing either test drugs or ritanserin controls. Data analysis was performed using ActivityBase (IDBS, Surrey, UK).

Behavioral Studies

Animals and Apparatus. Male non-Swiss Albino mice and male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were housed (four mice/cage; two rats/cage) in rooms with controlled temperature and humidity and freely available water and food (Harlan Teklad, Indianapolis, IN). Mice were kept on a 12-h light/dark cycle, whereas rats were kept on a 12-h reverse light/dark cycle. For locomotor and observation experiments in mice, plastic 20- \times 20- \times 30-cm activity cages were equipped with photocell beams (AccuScan Instruments, Columbus, OH). Startle chambers (San Diego Instruments, San Diego, CA) were used for rat experiments (for details on startle apparatus and measures, see Mansbach et al., 1988).

Procedures

Observation for Head Twitches. Mice were treated with 2.5 mg/kg DOI i.p. The dose of DOI was chosen based on pilot dose-effect curves, which revealed that the lowest doses consistently produced a significant behavioral effect. Five minutes later, mice were treated with AC-90179 s.c. and placed into activity cages. Ten minutes later, mice were observed using a repeated sampling technique. Each mouse was observed for 10 s and rated for presence (1) or absence (0) of head twitch behavior for a total of six observations in 15 min and a total head twitch score of 0 to 6. Each dose combination was tested in a separate group of animals ($n = 8$), and the experimenter was blind to drug conditions. Head twitch scores were averaged followed by analysis of variance (ANOVA) and post hoc Dunnett's *t* test comparisons.

Locomotor Activity. For hyperactivity experiments, mice were treated with 0.3 mg/kg dizocilpine or 3.0 mg/kg *d*-amphetamine i.p. 15 min before the session. The doses of *d*-amphetamine and dizocilpine were chosen based on pilot dose-effect curves, which revealed that the lowest doses consistently produced a significant behavioral

effect. Five minutes after pretreatment, mice were treated with AC-90179 s.c. and placed into the activity cages. For spontaneous activity, AC-90179 was administered alone. Locomotor data were collected during a 15-min session without habituation in a lit room. Each dose combination was tested in a separate group of animals ($n = 8$). Distance traveled (cm) was calculated and averaged followed by ANOVA and post hoc Dunnett's t test comparisons.

Startle Testing. Rats were tested and groups ($n = 10$) matched for levels of startle reactivity and prepulse inhibition (PPI; Mansbach et al., 1988). Two days later, test sessions started and consisted of a 5-min acclimation period with a constant background noise (65 dB), followed by 60 presentations of acoustic stimuli to measure acoustic startle responses. The 60 trials consisted of 22 40-ms presentations of a 120-dB broadband pulse, 10 20-ms presentations of each prepulse intensity (68, 71, and 77 dB) 100 ms prior to a 40-ms presentation of a 120-dB broadband pulse, and 8 NOSTIM trials in which no stimuli were delivered to assess general motor activation in the rats. Thirty minutes before testing, rats were treated with sterile water (s.c.), risperidone (1.0 mg/kg, i.p.), or AC-90179 (s.c.). Five minutes later, rats were administered DOI (0.5 mg/kg, s.c.) or 0.9% saline (s.c.). One week later, rats were administered the same pretreatment drug or vehicle and crossed over to receive the treatment opposite to that they received the previous week. Startle magnitudes and percentage of PPI for the three prepulse intensities were calculated as described elsewhere (Bakshi et al., 1994) and ANOVAs with repeated measures performed.

Results

As part of an ongoing effort to enable a universal functional assay for GPCR subtypes, we have cloned all of the known monoaminergic receptor subtypes and transiently expressed most of them in NIH-3T3 cells to determine their functional responses and pharmacological profiles for a large series of existing therapeutic agents. Data from concentration-response experiments with reference full agonist and full inverse agonist compounds for selected receptors are shown in Fig. 1. We focused our initial efforts on Gq-coupled receptors to take advantage of the observation that coexpression of the alpha subunit of Gq can be used to augment constitutive signaling (Burstein et al., 1997b), allowing the determination of inverse agonist pharmacology. The histamine H_1 , muscarinic m5, and serotonin 5-HT_{2B} and 5-HT_{2C} (VGV) receptors display minimal endogenous basal activity. The coexpression of Gq resulted in varying degrees of constitutive activity for these receptors (Fig. 1, A–D). In contrast, the 5-HT_{2A} receptor displayed modest degrees of basal activity (Fig. 1E). Consistent with previous observations in rat (Niswender et al., 1999), the human 5-HT_{2C} receptor displays a range of constitutive signaling from the minimal degree seen with the fully edited VGV isoform, the intermediate degree observed with the partially edited VSV isoform, to the profoundly activated unedited INI isoform of this receptor (Fig. 1, D, F, and G). We controlled for both endogenous receptor and non-receptor-mediated inhibition or promotion of cellular growth by assaying all compounds against cells expressing the β -galactosidase marker gene alone and cells expressing unrelated receptors. The constitutive activity of the various receptor subtypes demonstrated in this study, and the inverse agonist activity of the compounds tested, could have been the result of inclusion of trace amounts of agonists in the media used to culture the NIH-3T3 cells as part of the assay. To address this concern, we replaced calf serum with synthetic medium that is free of monoamines. To definitively prove that endog-

enous serotonin was not present, we also coexpressed the serotonin transporter (Blakely et al., 1994), which abolished all 5-HT-mediated signaling yet did not affect the inverse agonist responses observed at the 5-HT_{2A} receptor (data not shown).

A library of 640 clinically relevant compounds (Sigma/RBI) was screened for intrinsic activity at the three 5-HT₂ receptor subtypes and at m5CAM (Spalding et al., 1995). Known serotonergic agonists showed activity at only the three 5-HT₂ receptor subtypes, while known muscarinic agonists were selectively active at the m5 receptor. Interestingly, many dopaminergic compounds were identified as 5-HT_{2A} receptor agonists, including commonly used drugs such as pergolide and bromocriptine. Many serotonergic reference competitive antagonists including ritanserin, ketanserin, and methiothepin displayed inverse agonist activity at all three serotonin receptor subtypes, but not at the m5 receptor, where known muscarinic inverse agonists such as atropine, benztropine, and trihexyphenidyl were identified. Most compelling was the finding that nearly every known antipsychotic in this library was identified as a 5-HT_{2A} receptor inverse agonist (17/18 with raclopride as the exception). This result was not seen with the closely related 5-HT_{2B} or the 5-HT_{2C} receptors, where only a significantly smaller subset of these compounds was active. Four antipsychotics in this library (clozapine, loxapine, thioridazine, and chlorpromazine) were identified as muscarinic receptor inverse agonists, whereas most of the tricyclic antidepressants displayed this activity (data not shown).

A detailed pharmacological profiling of 40 antipsychotics was generated at these three 5-HT₂ receptors, as well as at the histamine H_1 and dopamine D_2 receptor. The results of these experiments are shown in Table 1. In general, the pharmacology of these compounds correlates well with published values derived from receptor binding techniques (Creese et al., 1976; Seeman et al., 1976; Leysen et al., 1978; Hals et al., 1988; Roth et al., 1992; Stockmeier et al., 1993). As expected, nearly all compounds tested were potent competitive antagonists of the dopamine D_2 receptor. Surprisingly, nearly all of these compounds were also potent 5-HT_{2A} receptor inverse agonists. The majority of these compounds were full inverse agonists (>90% relative efficacy compared with ritanserin), whereas those few compounds that displayed partial inverse agonist activity also displayed lower 5-HT_{2A} potencies. Of the six antipsychotics that lacked 5-HT_{2A} inverse agonist activity, five of these retained intrinsic activity at this receptor as very low-potency (>1 μ M EC₅₀) agonists, of which four belong to a single chemical class, the substituted benzamides. Only a single agent, perazine, was a potent partial agonist at this site, with an EC₅₀ of 140 ± 40 nM with 40% efficacy relative to serotonin. With the exception of tiapride, all of the remaining non-5-HT_{2A} inverse agonists display appreciable potency as D_2 receptor competitive antagonists (0.7–136 nM; Table 1). Extensive profiling of neuropsychiatric agents from different clinical and chemical classes has elucidated competitive antagonists lacking negative intrinsic activity at the 5-HT₂ receptors, yet all competitive antagonists of the histamine H_1 receptor behave as inverse agonists (data not shown). Lastly, broader profiling of these 40 antipsychotics against most of the remaining monoaminergic GPCRs did not reveal any other *in vitro*

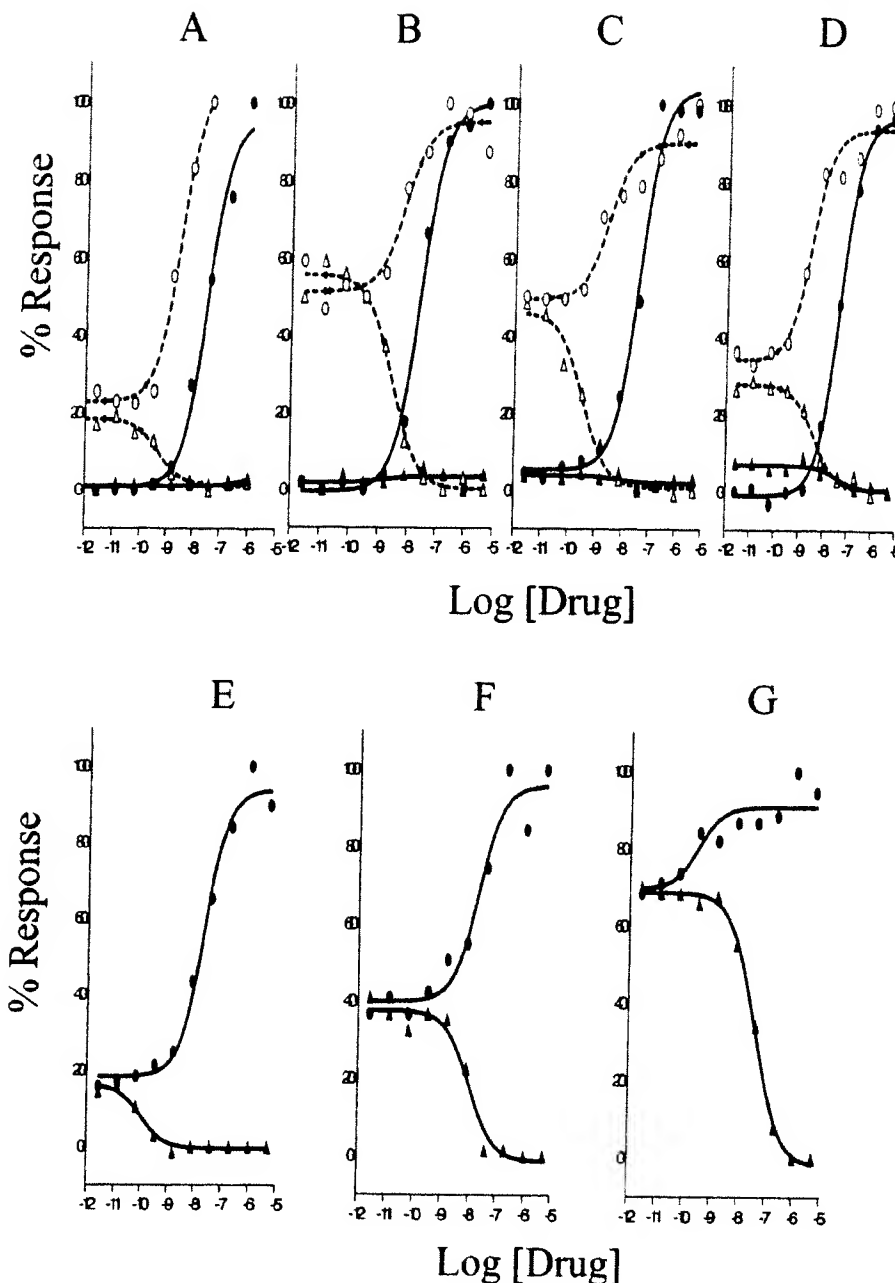


Fig. 1. Constitutive activity of monoamine receptors. Representative concentration response experiments for the human H₁ (A), m5 (B), 5-HT_{2B} (C), 5-HT_{2C}-VGV (D), 5-HT_{2A} (E), 5-HT_{2C}-VSV (F), and 5-HT_{2C}-IND (G) receptors as determined using R-SAT. The y-axis is the percentage of response defined as Full Agonist Response - Full Inverse Agonist Response; the x-axis is the negative logarithm of drug concentration. Curves with circles represent agonist determinations, while those with triangles represent inverse agonists. For those receptors without significant constitutive activity, coexpression of Gq was used, and the effects are shown with open circles (agonists) and open triangles (inverse agonists). For the m5 receptor, the m5CAM was used instead of Gq coexpression. Reference full agonists and full inverse agonists used were serotonin and ritanserin for 5-HT₂ receptors, histamine and nicpyramine for H₁ receptors, and carbachol and atropine for m5 CAM. EC₅₀ values for these receptors are reported as mean \pm standard deviation of three to eight replicate experiments and are as follows: H₁, 210 \pm 3.3 nM agonist; H₁ and Gq, 46 \pm 15 nM agonist, 3.3 \pm 1 nM inverse agonist; m5, 25 \pm 3 nM agonist; m5CAM, 8.3 \pm 2 nM agonist, 7.4 \pm 3 nM inverse agonist; 5-HT_{2A}, 29 \pm 12 nM agonist, 0.67 \pm 0.3 nM inverse agonist; 5-HT_{2B}, 37 \pm 8 nM agonist; 5-HT_{2B} and Gq, 9.2 \pm 7 nM agonist, 1.9 \pm 1 nM inverse agonist; 5-HT_{2C} (VGV), 42 \pm 8 nM agonist; 5-HT_{2C} (VSV), 12 \pm 5 nM agonist, 11 \pm 3 nM inverse agonist; and 5-HT_{2C} (IND), 2.1 \pm 2 nM agonist, 390 \pm 28 nM inverse agonist. The percentage of constitutive activity was determined by (Basal Response - Full Inverse Agonist Response) / (Full Agonist Response - Full Inverse Agonist Response) and were as follows: H₁, none; H₁ (+Q), 30 \pm 6%; m5, none; m5CAM, 51 \pm 2%; 5-HT_{2A}, 24 \pm 3%; 5-HT_{2B}, none; 5-HT_{2B} (+Q), 58 \pm 6%; 5-HT_{2C} (VGV), 2.6 \pm 1%; 5-HT_{2C} (VSV), 53 \pm 6%; and 5-HT_{2C} (IND), 87 \pm 5%.

molecular activity that correlates with efficacy of this class of compounds (unpublished observations).

A library of chemically diverse organic compounds was screened for 5-HT_{2A} receptor inverse agonists. All compounds were tested at a 3 μ M concentration, and ritanserin (100 nM) was used as a positive control. Of the 130,000 compounds tested in this assay, 530 initial hits were confirmed as 5-HT_{2A} inverse agonists. These compounds were subsequently tested at additional doses (3000, 300, 30, and 3 nM) to determine their relative potencies, and as inverse agonists at the m5 receptor to eliminate compounds that display nonspecific inhibitory responses. Of the confirmed hits in the initial screen, 230 compounds had potencies between 300 and 30 nM, while 96 compounds had potencies less

than 30 nM and were at least 100-fold selective for 5-HT_{2A} relative to m5. A number of chemical classes emerged, including two related to known butyrophenone and tricyclic antipsychotics, as well as multiple novel series containing piperidine and piperazine moieties. One such structural series was explored further by assaying 1400 additional analogs for 5-HT_{2A} receptor inverse agonist activity. Of these analogs, 176 compounds had activity at 3 μ M, of which 22 compounds had potencies less than 300 nM. Additional synthetic medicinal chemistry efforts led to the development of a defined structure-activity relationship with a number of potent analogs within multiple structural series. One lead compound, AC 90179 [$\log P_{\text{K}_a}$ of -8.82 ± 0.18 ($n = 10$)], the structure of which is shown in Fig. 2A, was chosen to undergo

TABLE 1

Monoamine receptor antagonist and inverse agonist activity of antipsychotics

All values represent the mean \pm the standard deviation, reported in nanomolar units, from three to eight replicate concentration response experiments. Dopamine D_2 receptor values are K_i determinations of these compounds as competitive antagonists using pergolide as the competing ligand, and all values are calculated by the formula $K_i = (IC_{50, \text{observed}} / (1 + [\text{agonist}] / EC_{50, \text{agonist}}))$. Most of the remaining values are EC_{50} determinations of these compounds as inverse agonists at these receptor subtypes, with the exception of pimozide and butaclamol, which are reported as K_i values at the 5-HT_{2A} receptor. These two compounds failed to display intrinsic activity at this receptor subtype, but are potent competitive antagonists when assayed against serotonin as the competing ligand. The numbers assigned to each antipsychotic are used for reference to Fig. 3.

Antipsychotic		5-HT _{2A}	D ₂	H ₁	5-HT _{2B}	5-HT _{2C}
Sertindole	1	0.1 \pm 0.03	5.2 \pm 3	1000 \pm 340	0.3 \pm 0.2	23 \pm 9
Octoclotheptin	2	0.2 \pm 0.02	0.12 \pm 0.6	2.8 \pm 0.4	0.5 \pm 0.01	3 \pm 1
M100907	3	0.3 \pm 0.08	1030 \pm 330	370 \pm 67	N.A.	460 \pm 94
Telfudazine	4	0.7 \pm 0.2	0.15 \pm 0.8	18 \pm 5	0.2 \pm 0.04	5 \pm 1
Spiroperone	5	1.6 \pm 0.4	0.06 \pm 0.04	1700 \pm 700	2.6 \pm 0.8	N.A.
Tiospirone	6	1.8 \pm 0.4	0.5 \pm 0.1	70 \pm 20	N.A.	470 \pm 98
Risperidone	7	1.8 \pm 0.3	6.1 \pm 4	4000 \pm 910	220 \pm 70	N.A.
Pimozide	8	2.2 \pm 0.1	0.2 \pm 0.2	51 \pm 11	27 \pm 6	194 \pm 76
Clothiapine	9	2.8 \pm 0.2	7.0 \pm 3	4 \pm 1	12 \pm 4	450 \pm 98
Amoxapine	10	3.6 \pm 0.8	15 \pm 8	46 \pm 9	45 \pm 21	120 \pm 45
Loxapine	11	4.2 \pm 0.9	1.8 \pm 0.5	5 \pm 1	30 \pm 9	100 \pm 170
Clozapine	12	6.4 \pm 1	73 \pm 48	0.4 \pm 0.1	9 \pm 2	250 \pm 58
Olanzapine	13	6.8 \pm 0.6	29 \pm 17	19 \pm 5	34 \pm 9	360 \pm 110
cis-flupentixol	14	7 \pm 1	0.2 \pm 0.1	8.3 \pm 1	9 \pm 2	1760 \pm 360
Fluspirilene	15	13 \pm 3	0.5 \pm 0.2	350 \pm 90	160 \pm 68	N.A.
Butaclamol	16	14 \pm 9	0.4 \pm 0.2	N.A.	N.A.	464 \pm 49
Chlorpromazine	17	16 \pm 4	3.6 \pm 3	47 \pm 8	260 \pm 67	N.A.
Amperozide	18	16 \pm 2	631 \pm 170	480 \pm 84	30 \pm 9	N.A.
Fluphenazine	19	17 \pm 5	0.1 \pm 0.06	45 \pm 9	82 \pm 23	N.A.
Chlorprothazene	20	20 \pm 9	3.4 \pm 2	130 \pm 37	1300 \pm 360	N.A.
Trifluoperidol	21	26 \pm 3	0.2 \pm 0.1	1900 \pm 520	260 \pm 23	N.A.
Perlapine	22	30 \pm 10	273 \pm 240	0.2 \pm 0.03	60 \pm 10	1300 \pm 88
Promazine	23	80 \pm 4	101 \pm 9	15 \pm 5	N.A.	N.A.
Moperone	24	87 \pm 13	1.5 \pm 0.3	1100 \pm 560	850 \pm 350	N.A.
Thioridazine	25	100 \pm 18	9.8 \pm 3	74 \pm 16	N.A.	N.A.
Mesoridazine	26	100 \pm 50	7.5 \pm 4	26 \pm 7	N.A.	N.A.
Melperone	27	110 \pm 30	82 \pm 34	N.A.	N.A.	N.A.
Trifluoperazine	28	130 \pm 45	9.3 \pm 0.7	370 \pm 82	N.A.	N.A.
trans-flupentixol	29	130 \pm 40	4.5 \pm 3.0	29 \pm 7	34 \pm 8	2500 \pm 700
Haloperidol	30	210 \pm 50	0.8 \pm 0.3	2900 \pm 990	N.A.	N.A.
Bromperidol	31	220 \pm 30	0.3 \pm 0.1	N.A.	N.A.	N.A.
Prothipendyl	32	250 \pm 91	294 \pm 150	32 \pm 8	N.A.	N.A.
Quetiapine	33	270 \pm 43	47 \pm 28	5.6 \pm 1	1100 \pm 120	N.A.
Thiothixene	34	320 \pm 48	0.1 \pm 0.06	16 \pm 4	N.A.	N.A.
Perazine	35	N.A.	136 \pm 54	34 \pm 8	N.A.	N.A.
Molindone	36	N.A.	4.4 \pm 2	N.A.	N.A.	N.A.
Remoxipride	37	N.A.	89 \pm 27	N.A.	N.A.	N.A.
Sultopride	38	N.A.	8.7 \pm 8	N.A.	N.A.	N.A.
Sulpiride	39	N.A.	0.7 \pm 0.4	N.A.	N.A.	N.A.
Tiapride	40	N.A.	573 \pm 320	N.A.	N.A.	N.A.

N.A., no activity as an inverse agonist.

receptor selectivity profiling. This compound was functionally profiled as both an agonist, and as either a competitive antagonist or inverse agonist, at 32 of the 35 known monoaminergic GPCRs (all known human receptor subtypes except the $\alpha_{1A/D}$, 5-HT_{5A}, and dopamine D₁ receptors). AC-90179 displays nearly 100-fold selectivity against the 5-HT_{2B} receptor [-6.87 ± 0.24 ($n = 9$)], 5-HT_{2C} (INI) [-6.94 ± 0.38 ($n = 10$)], and 5-HT_{2A} receptor [-6.8 ± 0.18 ($n = 2$)] as inverse agonists. This compound lacks activity (defined as $> 1 \mu\text{M}$ EC_{50}) at all other receptors tested (data not shown).

To characterize the behavioral profile of a selective 5-HT_{2A} receptor inverse agonist, AC-90179 was tested in head twitch, locomotor, and PPI behavioral models. DOI-treated (2.5 mg/kg, i.p., 15 min) mice exhibited an average head twitch score of 2.6 (± 0.3 S.E.M.). AC-90179 (0.1–30 mg/kg, s.c., 10 min) caused a dose-related decrease in DOI induced head twitches with a minimum effective dose of 1 mg/kg and with higher doses completely eliminating head twitch behavior (Fig. 2A). In the locomotor experiments (Fig. 2B), mice traveled an average of 794 cm (± 122 S.E.M.) after vehicle

administration. Dizocilpine (0.3 mg/kg, i.p., 15 min) and *d*-amphetamine (3.0 mg/kg, i.p., 15 min) caused increases in distance traveled with averages of 2625 (± 312) and 3367 (± 532) cm, respectively. AC-90179 (0.3–10 mg/kg, s.c., 10 min) attenuated the hyperactivity induced by dizocilpine, but not by *d*-amphetamine. The minimum effective dose against dizocilpine was 1 mg/kg, whereas AC-90179 reduced spontaneous locomotor activity only at the highest dose tested (30 mg/kg).

DOI significantly disrupted PPI, and AC-90179 was effective in restoring this disruption, especially at the higher doses. AC-90179 did not affect PPI on its own, with no significant effect of pretreatment ($p > 0.05$) on percentage of PPI. The ANOVA on the PPI data from the risperidone comparison also revealed a significant effect of treatment [$F(1,18) = 14.08$, $p < 0.01$] and a treatment by pretreatment interaction [$F(1,18) = 24.48$, $p < 0.01$]. As predicted, risperidone was also effective in restoring PPI in DOI-treated rats, while having no effect on PPI by itself ($p > 0.05$). Since there were no significant interactions with prepulse intensity, the data were collapsed across the three prepulse intensities for

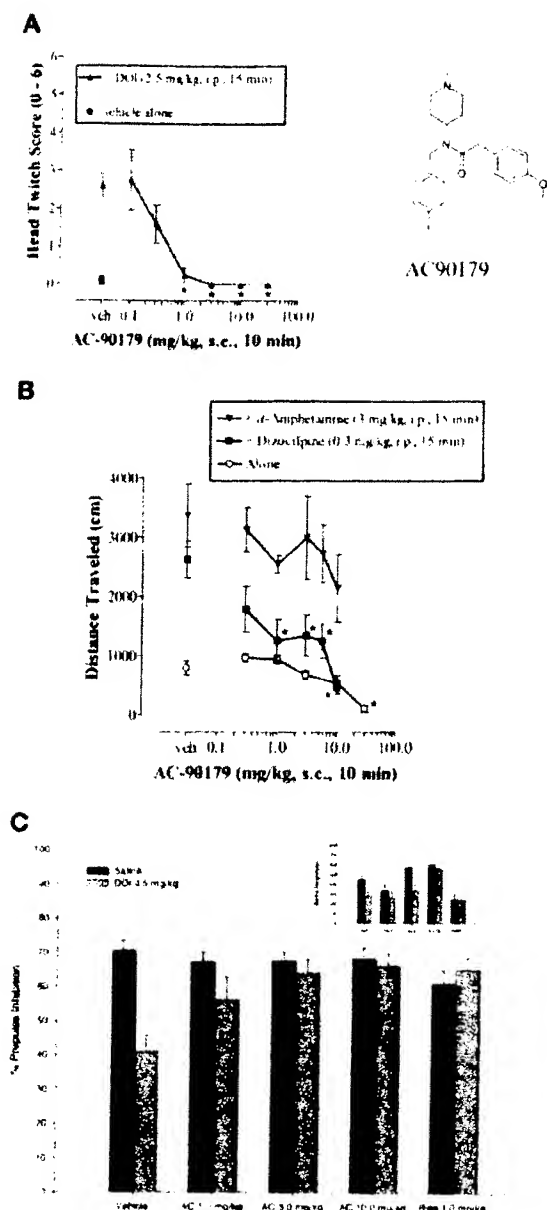


Fig. 2. Antipsychotic-like behavioral effects of AC-90179. **A**, head twitch score as a function of AC-90179 dose, and the chemical structure of AC-90179. Filled triangles, AC-90179 or vehicle in combination with 2.5 mg/kg DOI; filled circle, vehicle dosed alone. Each point represents the mean ($n = 8-24$), and vertical lines represent standard error of the mean. Asterisks indicate statistical significance ($p < 0.05$) compared with the respective vehicle control. **B**, distance traveled (cm) in the locomotor cages as a function of AC-90179 dose. Filled inverted triangles, AC-90179 or vehicle in combination with 3 mg/kg *d*-amphetamine; filled squares, AC-90179 or vehicle in combination with 0.3 mg/kg dizocilpine; open circles, AC-90179 or vehicle alone. Each point represents the mean ($n = 8-24$), and vertical lines represent standard error of the mean. Asterisks indicate statistical significance ($p < 0.05$) compared with the respective vehicle control. **C**, percentage of prepulse inhibition as a function of AC-90179 pretreatment dose or vehicle and risperidone pretreatment controls. Black columns, the means of saline treated rats; gray columns, DOI (0.5 mg/kg)-treated rats. Error bars represent the S.E.M. The ANOVA on the G-PPI data from the AC-90179 groups revealed an overall effect of treatment [$F(3,37) = 27.73, p < 0.01$] and a treatment by pretreatment interaction [$F(3,37) = 8.22, p < 0.01$] (*). The inset shows the effects on startle magnitude by AC-90179 or risperidone in saline or DOI-treated rats.

graphical purposes (Fig. 2C). Since there was a significant pretreatment by treatment interaction, pair-wise 2-way repeated measures ANOVAs were conducted on the saline- and DOI-treated groups. In the vehicle-treated rats, there was no effect of AC-90179 ($p > 0.025$) or risperidone ($p > 0.025$) on PPI. In the DOI-treated groups, there were significant effects of AC-90179 [$F(3,37) = 5.68, p < 0.01$] and risperidone [$F(1,18) = 16.73, p < 0.01$] on percentage of PPI. The ANOVA on startle magnitude from the AC-90179 groups revealed significant effects of pretreatment [$F(3,37) = 2.89, p = 0.048$] and treatment [$F(1,37) = 10.27, p < 0.01$] on startle magnitude, but no treatment by pretreatment interaction ($p > 0.05$; Fig. 2C, inset). Risperidone, on the other hand, had no effect on startle magnitude ($p > 0.05$).

Discussion

These data represent the first description of the functional profile of a large set of antipsychotics at multiple receptor subtypes. Two mechanistic correlations have emerged from this analysis. Potency values from the R-SAT assay reconfirm the previously reported correlation between D₂ receptor competitive antagonism and antipsychotic efficacy and establish a correlation between inverse agonism at 5-HT_{2A} receptors and efficacy that is nearly as complete. No such correlation can be made between these compounds and the highly homologous 5-HT_{2B} and 5-HT_{2C} receptor subtypes, or with any other monoamine receptor subtype tested to date. The compounds that fail to display 5-HT_{2A} inverse agonist activity (i.e., substituted benzamides) have relatively potent D₂ receptor competitive antagonist activity. Taken together, these data argue that competitive antagonism of D₂ receptors and inverse agonism of 5-HT_{2A} receptors are independent mechanisms of antipsychotic efficacy.

While a number of groups have heterologously expressed 5-HT_{2A} receptors, none has been able to determine whether classic receptor competitive antagonists actually possess negative intrinsic activity at this receptor (Leysen et al., 1978; Roth et al., 1992; Stockmeier et al., 1993; Kehne et al., 1996; Grotewiel and Sanders-Bush, 1999). Mutagenic studies have yielded 5-HT_{2A} receptors that display constitutive activity, and a small number of antipsychotics were shown to possess partial inverse agonist activity (Egan et al., 1998a,b). However, the limited pharmacology conducted on these mutated receptors precluded the ability to recognize inverse agonism of 5-HT_{2A} receptors as a common efficacy mechanism of this class of compounds. That many antipsychotics are potent 5-HT_{2A} receptor competitive antagonists has been appreciated for some time (Leysen et al., 1978), and subsequent investigations into the mechanism of action of clozapine and related atypical agents have indicated the importance of mixed 5-HT_{2A} and dopamine D₂ receptor competitive antagonism (Stockmeier et al., 1993). Since the clinical dosing of antipsychotics is often limited by their D₂ receptor-induced extrapyramidal side effects, aspects of their clinical profiles can be predicted based on their in vitro potency ratios using this receptor as a basis for comparison. Figure 3 graphically depicts the in vitro potencies of these 40 compounds as inverse agonists of the 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, and H₁ receptor subtypes compared with their potencies as competitive dopamine D₂ receptor antagonists. Many of these compounds are selective (defined as > 10 -fold relative potency) for the D₂

receptor over all other sites. These D_2 -selective compounds, including haloperidol, thiothixene, and fluphenazine, have the highest propensity to induce extrapyramidal side effects. None of the atypical agents displayed a preference for D_2 , as they were either equipotent at D_2 and 5-HT_{2A} , or preferred 5-HT_{2A} sites (Fig. 3A). Only three compounds, sertindole, M100907, and amperozide, displayed 5-HT_{2A} receptor selectivity compared with D_2 . While these compounds have been shown to possess antipsychotic efficacy, and two are widely considered to be atypical, their clinical profiles are currently not well established (Axelsson et al., 1991; van Kammen et al., 1996). This analysis confirms not only the critical role of 5-HT_{2A} receptors in defining atypicality of an antipsychotic (Stockmeier et al., 1993), but also shows that a small number of existing clinically efficacious agents are selective 5-HT_{2A} inverse agonists. Compounds with H_1 receptor selectivity, namely clozapine and perlapine (Fig. 3C), are known to produce significant sedation in routine clinical use, and are often used in agitated patients with prominent sleep disturbances. That so many antipsychotics possess H_1 receptor inverse agonist properties, yet only a small subset of these cause weight gain, argues against this site being responsible for this particular side effect of these drugs. Interestingly, not a single compound tested was selective for the 5-HT_{2C} receptor (Fig. 3D). Finally, these data do not support the recent hypothesis that inverse agonism of 5-HT_{2C} receptors defines an atypical clinical profile (Herrick-Davis et al., 2000).

The present observations lend support to the hypothesis that selective 5-HT_{2A} inverse agonists devoid of D_2 receptor competitive antagonism will have antipsychotic efficacy in humans. Since chronic blockade of D_2 receptors is responsible for severe motor and cognitive side effects, compounds that can maintain antipsychotic efficacy without D_2 blockade would probably result in novel agents with truly unique clinical features. Although clinical experience with selective 5-HT_{2A} inverse agonists is limited, sertindole and amperozide

appear to have a lower liability for these side effects than even the mixed atypical agents (Axelsson et al., 1991; van Kammen et al., 1996). One could speculate that selective 5-HT_{2A} inverse agonists will also have unique efficacy profiles, with distinct advantages in maintenance therapy, and perhaps improved efficacy in patient subgroups that display heightened serotonergic or diminished glutaminergic tone (see discussion below). That AC-90179 attenuated DOI-induced head twitches in mice and PPI disruptions in rats is consistent with a 5-HT_{2A} receptor mechanism of action in vivo and with antipsychotic-like efficacy. The attenuations by AC-90179 of both the hyperactivity and the disruption of PPI produced by dizocilpine are similar to the effects observed with M100907 (Martin et al., 1997; Varty et al., 1999). That AC-90179 attenuated dizocilpine-induced but not amphetamine-induced hyperactivity is consistent with an atypical-like antipsychotic profile. Furthermore, the 30-fold potency separation between activity against DOI or dizocilpine-induced effects in mice and spontaneous locomotor activity in rats suggest a wide therapeutic index for efficacy versus motor side effects. These data support the notion that selective 5-HT_{2A} receptor inverse agonists, such as AC-90179, will be efficacious and lack the side effects of compounds in current use.

The observation that some existing therapeutic agents possess negative intrinsic activity at various receptors raises intriguing possibilities as to the role of constitutive receptor activity in vivo. Studies with transgenic mice that were designed by overexpression to increase adrenergic receptor tone in their cardiovascular systems have revealed that β -blockers with negative intrinsic efficacy are physiologically distinct from those that lack this molecular property (Bond et al., 1995; Nagaraja et al., 1999). Native histamine H_3 receptors display significant constitutive activity in vivo, and physiological distinctions between competitive antagonists and inverse agonists were noted (Morisset et al., 2000). Pre-

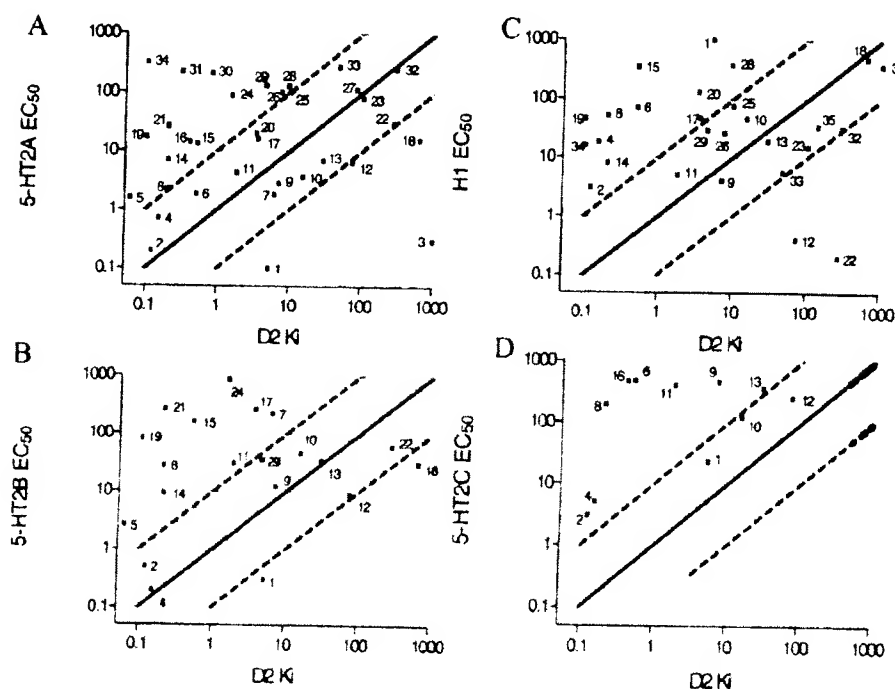


Fig. 3. Correlations of antipsychotic activity at D_2 versus 5-HT_{2A} , 5-HT_{2B} , 5-HT_{2C} , and H_1 receptors. Comparison of the potency of a series of antipsychotics as D_2 receptor competitive antagonists versus 5-HT_{2A} , 5-HT_{2B} , 5-HT_{2C} , and H_1 receptor inverse agonists. Inverse agonist values are expressed as EC_{50} values, while competitive antagonist values are expressed as K_i values. All values are in nanomolar units and are derived from data presented in Table 2. Filled line, a one to one correlation in receptor potencies; dashed lines, 10-fold selectivity for D_2 or the various monoamine receptors, respectively. A, the 5-HT_{2A} receptor; B, the 5-HT_{2B} receptor; C, the histamine H_1 ; and D, the 5-HT_{2C} receptor. All compounds are coded according to their designation in Table 2, where any with potencies above $1 \mu\text{M}$ were excluded from the analysis.

vailing theories as to the pathophysiological basis of schizophrenia center on the finding that antipsychotics are D₂ and 5-HT_{2A} receptor competitive antagonists. Results of studies designed to document elevated dopaminergic or serotonergic neurotransmitter levels in the central nervous system of schizophrenics have been mixed to date. The present findings argue that levels of constitutive receptor activity may be the critical determinant of neurotransmitter tone in the central nervous system. Recent studies concerning the role of 5-HT_{2A} receptors and their relationship to glutamatergic systems have concluded that elevated 5-HT_{2A} receptor tone, and the attenuation of such tone, is a critical property for efficacy as an antipsychotic (Martin et al., 1998). Similarly, as the number of human disorders known to be caused by dominant mutations in GPCRs increases (Birnbauer, 1995), it becomes reasonable to suggest that activating mutations in 5-HT_{2A} receptors may be causative or predisposing to neuropsychiatric disease, or modulate response to treatment with antipsychotics (Arranz et al., 2000). Lastly, the creation of a transgenic mouse model that exhibits increased 5-HT_{2A} receptor activity may provide an excellent preclinical model of antipsychotic efficacy.

The discovery of novel therapeutic compounds used to occur without knowledge of an actual molecular target, and it was frequently based on the serendipitous observation of clinical efficacy of a parent chemical structure. Antipsychotics are a good example, as the prototypical agent chlorpromazine was developed as an anesthetic adjuvant, but later was found to be effective in the management of human psychoses (Deniker, 1990). A similarly serendipitous path led to the initial discovery of antidepressants, anxiolytics, and mood stabilizers. We have explored the functional nature of the interaction between existing drugs and many of their potential targets. This evidence-based approach has elucidated the importance of inverse agonism at an established target as an efficacy mechanism for antipsychotics and has led to a successful drug discovery effort that exploits the observation of negative intrinsic activity of GPCRs. Similar evidence-based approaches to related agents like antidepressants may lead to the development of improved therapeutics for many neuropsychiatric disorders.

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EXHIBIT

10A – 10F

5-HT_{2B} receptors play a key role in mediating the excitatory effects of 5-HT in human colon *in vitro*

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1 5-Hydroxytryptamine (5-HT) is known to produce a number of different effects in the gastrointestinal tract of various species, and has been proposed to play a key role in a number of intestinal disorders in man, including irritable bowel syndrome (IBS), although the receptors involved have yet to be established. The aim of the present study was to investigate the distribution and function of 5-HT_{2B} receptors in human colon, and to establish their possible role in the aetiology of IBS.

2 The distribution of 5-HT_{2B} receptor mRNA and protein were investigated by quantitative RT–PCR, Western analysis and immunocytochemistry. High levels of both mRNA and protein for 5-HT_{2B} receptors were found throughout the human gastrointestinal tract, and in particular in colon, where 5-HT_{2B} receptors were found predominantly in the longitudinal and circular smooth muscle layers within the muscularis externa, and in the myenteric nerve plexus lying between these two layers.

3 Electrical field stimulation of longitudinal muscle preparations of human colon mounted in organ baths resulted in neuronally-mediated contractile responses, that were significantly potentiated by application of 5-HT (up to 10^{–7} M), with a pEC₅₀ of 8.2 ± 0.1 (*n* = 49 donors). The response to 5-HT was inhibited by a number of selective 5-HT_{2B} receptor antagonists.

4 This study has shown for the first time that, in contrast to animal studies, the excitatory effects of 5-HT in human colon are mediated by 5-HT_{2B} receptors. It is proposed that these receptors contribute to the putative 5-HT-induced colonic smooth muscle hypersensitivity associated with IBS. *British Journal of Pharmacology* (2002) **135**, 1144–1151

Keywords: 5-Hydroxytryptamine; 5-HT_{2B}; human colon; irritable bowel syndrome; motility disorders

Abbreviations: 2-Me-5-HT, 2-methyl-5-hydroxytryptamine; 5-MeOT, 5-methoxytryptamine; 5-HT, 5-hydroxytryptamine; 5-HT_{2B} R-ir, 5-HT_{2B} receptor-like immunoreactivity; α -Me-5-HT, alpha-methyl-5-hydroxytryptamine; BSA, Bovine serum albumin; CR, Concentration-ratio; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IBS, Irritable bowel syndrome; PBS, Phosphate-buffered saline; RS-127445, 2-amino-4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine; RT–PCR, Reverse-transcriptase polymerase chain reaction; SB-204741, N-(1-methyl-5-indolyl)-N'-(3-methyl-5-isothiazolyl)urea; SB-206553, 5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-f]indole; SB-242084, 6-chloro-5-methyl-1-[6-(2-methylpyridin-3-yloxy)pyridin-3-yl-carbamoyl]indoline

Introduction

Physiological and pharmacological studies have indicated that 5-HT may play a pivotal role in mediating sensory and reflex responses in the gastrointestinal tract of various species, including man (Read & Gwee, 1994). Whilst the receptors responsible for these effects in animals have been extensively investigated and subsequently identified, relatively little is known of the 5-HT receptor sub-types responsible for the effects of this amine in the human intestine generally, and in human colon in particular.

Although it is generally accepted that the human small intestine contracts in response to 5-HT, it has been proposed that the predominant response in human colon is relaxation. In human ileum, application of 5-HT induces contraction of both circular and longitudinal muscle layers, *via* activation of 5-HT_{1D} and 5-HT_{2B} receptors respectively (Borman &

Burleigh, 1995; 1997a). In colon circular muscle, however, 5-HT has been shown to induce both smooth muscle relaxation and inhibition of spontaneous contractions, responses attributed to activation of 5-HT₄ and/or 5-HT₇ receptors (Borman & Burleigh, 1994; Prins *et al.*, 1999; Tam *et al.*, 1994). The response of longitudinal muscle strips to 5-HT is more complex, with some investigators reporting contraction and others reporting relaxation or a combination of contraction and relaxation (Hillier *et al.*, 1994). Although a recent study has indicated that 5-HT-induced excitatory effects in muscle strips of human colon *taenia coli* may be mediated by a 5-HT₄ receptor (Prins *et al.*, 2000), the receptors responsible for the effects in colon inter-taenial longitudinal smooth muscle have not previously been identified.

In addition to effects on smooth muscle activity, application of 5-HT induces profound effects on fluid secretion throughout the human intestine. Exogenous application of 5-HT has been shown to increase chloride ion (and thereby fluid) secretion in human jejunum, ileum and colon, the effect

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being more pronounced in small than large intestine (Borman & Burleigh, 1997b). In both human jejunum and ileum, and to a lesser extent proximal colon, the 5-HT₄ receptor has been shown to mediate the pro-secretory actions of 5-HT, resulting in significant ion and fluid accumulation into the lumen of the intestine (Borman & Burleigh, 1993; 1996; Budhoo *et al.*, 1996). Such activity has been proposed as a possible cause of hypersecretory disorders such as carcinoid syndrome and cholera toxin-induced diarrhoea, which have been associated with increased levels of intestinal 5-HT (Bearcroft *et al.*, 1996).

The range of its biological activities suggests that 5-HT could be involved in a number of pathological events in the gastrointestinal tract, although its physiological role is uncertain. In particular, alterations in the levels of 5-HT are believed to be responsible for the complex symptomatology of the irritable bowel syndrome (IBS). IBS is a disorder that is characterized by abdominal pain coupled to constipation, diarrhoea, or both. The underlying cause of IBS has not been elucidated. However, the diversity of actions of 5-HT in the intestine, including the possibility that 5-HT may play a role in visceral hyperalgesia (Sanger, 1996), coupled to the finding that increased levels of 5-HT and its metabolites have been found in the plasma of IBS patients (Bearcroft *et al.*, 1998), have prompted the hypothesis that the amine may play a key role in the aetiology of the disorder (see Gershon, 1999, for example).

Given the potential role of 5-HT in the pathophysiology of IBS, the aim of the present study was to investigate the nature of the 5-HT receptor(s) responsible for the excitatory effects of 5-HT in human colon smooth muscle. In particular, we have investigated the expression of mRNA and protein, as well as function, of 5-HT_{2B} receptors, and have identified a novel role for this receptor in the control of human colonic function.

Methods

Gene expression studies

Primer probe design The target DNA sequence for the 5-HT_{2B} receptor was retrieved from GenBank, and primer/probe sets were designed using Primer Express (Perkin Elmer ABI software) to amplify a small fragment of the target sequence. The primer/probe sequences were subjected to homology searches against GenBank to confirm that they were specific for the target amplicon. Forward primer: ACGCCTAACATGGTTGACTGTGTC; Reverse primer: TGAGGCTCTCTGTTCTGTTGGAA; Probe: AGGTGGC-AATGCTGGATGGTTCTCGA.

An assay system has been developed that allows the determination of the abundance of more than one mRNA species in a single tube by using probes for the two genes that have different fluorophores, which are spectrally distinct. This was applied to measure expression of the target gene together with the expression of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, to monitor RNA integrity. The primer/probe set for GAPDH was positioned so that it spans an exon/exon boundary and will therefore only be amplified from cDNA.

RNA isolation Total RNA was isolated from whole sections of different regions of human intestine, from three different donors, using Trizol™ according to the manufacturer's instructions. The RNA was subjected to a range of quality control checks. Samples were only used for transcription profiling if: (1) The ratio of the optical density (OD) readings for the sample determined at wavelengths of 260 nm and 280 nm was >1.7 indicating that levels of contaminating protein and/or phenol were low; (2) The sample had intact 18 s ribosomal RNA bands as determined by denaturing agarose gel electrophoresis; (3) Actin mRNA transcript levels were above 6000 copies per 100 ng of total RNA as determined by quantitative PCR (QRT-PCR); and (4) QRT-PCR was also used to determine the levels of genomic DNA within the sample. Samples were only used where the level of DNA formed less than 10% of the total nucleic acid.

Quantitative RT-PCR Copy numbers of mRNA for the 5-HT_{2B} receptor were determined by QRT-PCR using the ABI Taqman sequence detection system. Prior to reverse transcription the total RNA samples were treated with RNase-free DNase to remove any contaminating genomic DNA. In order to ascertain whether any DNA contamination remained following the DNase treatment, all samples were subjected to PCR amplification in the absence of reverse transcriptase. The RNA that had been DNase-treated was then annealed to reverse primers for the target and GAPDH genes. This was carried out in the presence of buffer and the sample was heated to 72°C and then cooled to 55°C. The reverse transcription was carried out by adding MuLV reverse transcriptase and nucleotides to the reaction. This was incubated for 30 min at 37°C to allow synthesis to take place. The sample was then heated to 90°C for 5 min to denature the enzyme. Quantitative sequence detection for both targets was carried out simultaneously on the resulting cDNA that had been made from 100 ng of total RNA. Forward and reverse primers and probes for target and GAPDH were added along with nucleotides, buffer and AmpliTaq Gold™ Taq polymerase. The PCR reaction took place under the following reactions conditions: 94°C for 12 min, followed by 40 cycles of 94°C for 15 s and 60°C for 30 s.

Determination of mRNA expression levels of 5-HT_{2B} receptors in human intestine Following extraction, the levels of mRNA for 5-HT_{2B} receptors were determined in full-thickness preparations of each of the following tissues: oesophagus, stomach (antrum, body, fundus and pylorus), liver, pancreas, gallbladder, duodenum, jejunum, ileum, caecum, colon and rectum. The study was repeated in tissues from three separate donors.

Protein expression studies

Preparation of human tissue extracts Snap frozen samples of human tissue (approximately 1 g each piece) were added to 10 volumes of ice-cold 50 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 0.2 mM PMSF. The tissue was homogenized using an Ultra-Turrax homogenizer on full speed for 10 s. Sodium dodecyl sulphate (SDS) was added at a final concentration of 1%, for 10 min at room temperature, to solubilize proteins. Insoluble material was removed by centrifugation at 1000 × *g* for 10 min at room temperature.

The supernatant was decanted and re-centrifuged at $40,000 \times g$ for 15 min at room temperature. Protein was determined by the BCA method using BSA as a standard. The final protein extracts were stored at -20°C until use.

Dot-blotting of 5-HT_{2B} receptor protein The level of 5-HT_{2B} receptor protein expression was determined in protein extracts from human ileum or colon. In brief, protein extracts were diluted to 0.4 mg protein/ml in 62.5 mM Tris-HCl (pH 6.8), 1% (w v⁻¹) SDS. Immunoblotting was performed using a 96-well perspex-blotting manifold (Life Technologies). Before use, the manifold was washed in detergent, and rinsed in distilled water. A nitrocellulose sheet, pre-soaked with distilled water and a buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol, was loaded into the manifold and clamped in place by vacuum pressure. Protein samples (100 μl well⁻¹) were added to wells and incubated for 1 h at room temperature.

The resultant protein blot was removed from the manifold and rinsed in phosphate-buffered saline (PBS) for 5 min. The blot was stained with Ponceau S for 15 min to visualize the protein bound and to inactivate endogenous alkaline phosphatase enzyme. Destaining was achieved using PBS before blocking in PBS containing 1% (w v⁻¹) Marvel, 1% (w v⁻¹) BSA, 1% (v v⁻¹) sheep serum for 1 h at room temperature. Anti-5-HT_{2B} antibody (Pharmingen) diluted to 0.1 μg ml⁻¹ in PBS, 1% Marvel, 0.25% BSA (reagent diluent) was added to the blot and incubated for 90 min at 37°C . After thorough washing, bound antibody was localized using an anti-mouse fluorescein-linked secondary antibody (1:600; Amersham) and an anti-fluorescein alkaline phosphatase conjugate (diluted in 1:2500 in Tris-buffered saline, 1% Marvel, 0.25% BSA). Finally, bound conjugate was localized using enhanced chemifluorescence and imaged using a fluorimager (STORM; Molecular Dynamics). The resultant image was quantified using ImageQuant software. Control values obtained using non-immune mouse IgG as the primary antibody were subtracted from the results for the anti-5-HT_{2B} antibody to correct for endogenous fluorescence or residual alkaline phosphatase activity in protein extracts. Western analysis confirmed the specificity and affinity of the antibody for the human 5-HT_{2B} receptor.

Immunocytochemistry studies

Tissue preparation Fresh frozen (10 μm) or formalin-fixed paraffin-embedded (5–7 μm) sections of human colon were mounted onto silane-coated slides. Frozen sections were stored at -80°C until use, while paraffin-embedded sections were stored at room temperature (RT). On the day of the study, frozen sections were brought to RT and air-dried for 1 h. These sections were fixed in acetone for 30 min and air-dried for 1 h. Paraffin-embedded sections were rehydrated in graded alcohols.

Immunocytochemistry Anti-5-HT_{2B} receptor antibody (IgG₁ isotype) was purchased from Pharmingen Inc., U.S.A. (Catalogue number 60531A, Lot number MO17437). All incubations and washes were carried out on an orbital shaker at RT and all washes were in phosphate-buffered saline (PBS) unless otherwise stated. Endogenous peroxidase activity was quenched by incubation in 1% H₂O₂ : 0.1% NaN₃ in PBS for

frozen sections, and 3% H₂O₂ in distilled water for paraffin-embedded sections (30 min each). At this stage, an antigen retrieval step was performed on paraffin-embedded sections. These sections were microwaved in 0.01 M citrate buffer for 20 min followed by cooling slowly in tap water. All sections were then incubated for 30 min with 10% blocking serum (Vector Universal Elite Kit). Subsequently, sections were incubated with the 5-HT_{2B} receptor antibody (1–2 μg ml⁻¹ in PBS) for 16 to 72 h at 4°C . Control sections were incubated with mouse IgG at 1–2 μg ml⁻¹. Sections were then washed (2 \times 5 min) and incubated with a biotinylated universal secondary antibody (Vector Universal Elite Kit) for 30 min, followed by washing (2 \times 5 min) and incubation with Vectastain Elite ABC reagent (Vector Universal Elite Kit) for 30 min. Subsequently, sections were incubated with 3',3'-diaminobenzidine tetrachloride (0.025% w v⁻¹) : 2O₂ (0.02% v v⁻¹) in 0.05 M Tris buffer (pH 7.6) for 5 min, followed by washing in distilled water. Sections were then counterstained in Mayer's haematoxylin (1 min), dehydrated to xylene and cover-slipped with DPX mountant (BDH Laboratories). Immunostained sections were viewed with a Zeiss Axioplan2 microscope.

Pharmacology

Sections of human colon were cut open along their longitudinal axis. The sections were pinned out flat and the mucosa carefully removed using sharp dissecting scissors. Once the mucosa was removed, the section was turned over to reveal the three *taenia coli* (*taenia mesocolica*, *taenia omentalis* and *taenia libera*) and the muscle bands that lie between them. Longitudinal muscle strips (2 mm wide by 20 mm long) were then cut from the tissue between the *taenia coli* and suspended between stainless steel hooks in organ chambers containing oxygenated (95% O₂ : 5% CO₂) Krebs solution at 37°C . The composition of the Krebs solution was as follows in mM: NaCl 118.2, KCl 4.69, MgSO₄·7H₂O 1.18, KH₂PO₄ 1.19, glucose 11.1, NaHCO₃ 25.0, CaCl₂·6H₂O 2.5.

Tissues were placed under a tension equivalent to 10 mN and left to equilibrate for a period of at least 60 min. Responses were recorded using isometric transducers coupled to an Apple Macintosh computer via a MacLab interface. After 60 min, the longitudinal muscle sections of human colon were stimulated electrically (at sub-maximal voltage with 60 s between successive stimulations) using parallel platinum wire electrodes and a Multistim D330 pulse stimulator. Upon electrical stimulation, the strips of human colon longitudinal smooth muscle responded with a rapid contraction. Tissues were stimulated for 10 s every 60 s, at 15 V and with a 1 ms pulse width, at a range of frequencies from 0.1 to 40 Hz (with at least 3 min at each frequency). After a maximum response, stimulation was halted and the tissues washed three times, with 5 min between, and left to equilibrate for 30 min. After this time, electrical stimulation was recommenced, using the same stimulation parameters as previously and a frequency that produced sub-maximal contractions, and was allowed to stabilize. Once the response to electrical stimulation had stabilized (stimulated responses differed by no more than 10%), the strips were exposed to increasing concentrations of 5-HT (or 5-HT receptor agonists, cumulative concentration-effect curves with a minimum of 3 min contact time at each concentration), in

the absence or presence of selective receptor antagonists (incubated for 30 min prior to exposure to 5-HT). In this way, a single concentration-effect curve to 5-HT was generated in each preparation, either in the absence or presence of antagonist. A concentration ratio (CR) was therefore generated between the EC₅₀ values for 5-HT in the absence and presence of antagonist, in tissue from the same donor.

Data analysis

To calculate antagonist pK_B values, the mean CR was plotted as log₁₀ (CR-1) against log antagonist molar concentration according to the method of Arunlakshana & Schild (1959). If the slope of the plot did not differ significantly from one, it was constrained to unity to calculate an apparent pK_B value. Where only one concentration of antagonist had been tested, apparent pA₂ values were calculated for individual data points according to the method of Mackay (1978). Where appropriate, statistical comparisons were carried out by ANOVA or Student's *t*-test, with *P* < 0.05 being taken to indicate statistical significance.

Human tissues

All samples of human tissue were obtained through medically qualified intermediaries with the informed consent of the donor or donor's next of kin, and with approval of the local research ethics committee. The tissues were transported to Pharmagene in phosphate-buffered saline solution on ice. For mRNA expression studies, sections were obtained from the region of intestine indicated from multi-organ donors (*n* = 23 donors), or from sections of intestine removed at operation for carcinoma (*n* = 6 donors). All tissues from multi-organ donors were obtained *via* intermediaries who had obtained from the donor's next of kin the express permission for the use of those tissues for research purposes. The age range for these donors was 17–79 years, with mean age (\pm s.e.mean) of 44.8 ± 2.4 years. For immunohistochemistry studies, sections of distal (descending) colon were obtained from specimens removed at operation for carcinoma *in situ* of the rectum.

For organ bath studies, sections of colon (10 ascending and 39 descending or sigmoid) were obtained from 49 donors, 26 male and 23 female, age range 40–96 (mean age \pm s.e.mean of 66.9 ± 1.8 years), undergoing operations for carcinoma (*n* = 39), diverticular disease (*n* = 4), Crohns disease (*n* = 1), ulcerative colitis (*n* = 3) or polyps (*n* = 2). For these studies, tissues were kept at 4°C until the experiment, which was carried out within 24 h of removal of the tissue from the patient. In all cases, the section of colon was judged to be macroscopically normal by a consultant histopathologist.

Materials

In addition to the above, the following chemicals were used for this study: alpha-methyl-5-hydroxytryptamine (α -Me-5-HT; Tocris Cookson), 5-hydroxytryptamine creatinine sulphate (RBI), 2-methyl-5-hydroxytryptamine maleate (2-Me-5-HT; RBI), 5-methoxytryptamine hydrochloride (5-MeOT; RBI), cisapride (synthesized in-house), RS-127445 (2-amino-4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine; synthesized in-house), SB-206553 (5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-*f*]indole; synthesized in-house), rau-wolscine hydrochloride (RBI), yohimbine hydrochloride (RBI), methiothepin maleate (Tocris Cookson), SB-204741 (N-(1-methyl-5-indolyl)-N'-(3-methyl-5-isothiazolyl)urea; synthesized in-house), SB-242084 (6-chloro-5-methyl-1-[6-(2-methylpyridin-3-yloxy)pyridin-3-yl-carbamoyl]indoline; synthesized in-house), ketanserin tartrate (RBI) and methysergide maleate (Tocris Cookson).

Results

Gene expression

In sections of human gastrointestinal tract, each obtained from three independent donors, 5-HT_{2B} receptor mRNA was expressed in all tissues tested (Figure 1). There were no significant differences in mRNA expression levels between different regions of the GI tract.

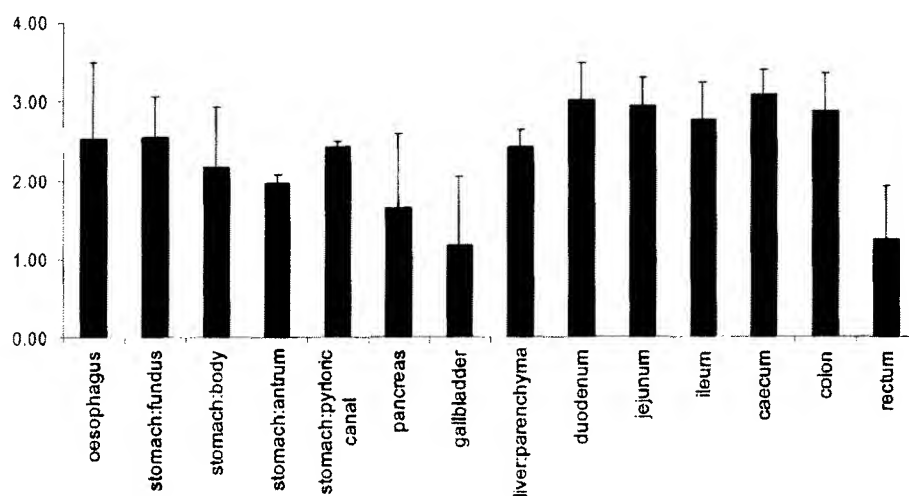


Figure 1 Expression of mRNA for 5-HT_{2B} receptors in human gastrointestinal tract. Data are expressed as mean log copy numbers (per 100 ng of total RNA) from three donors, with standard errors indicated by bars.

Protein expression

5-HT_{2B} receptors were detected in both human ileum and colon. In colon, highest expression was seen in smooth muscle, with little or no protein expression detected in the mucosa or *taenia coli* (Figure 2, data from a single donor). A similar pattern of expression was seen in ileum.



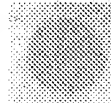






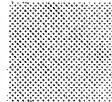




Tissue	Whole	Mucosa	Smooth muscle	<i>Taenia Coli</i>
Ileum 5-HT _{2B}				NA
Ileum IgG1				NA
Colon 5-HT _{2B}				
Colon IgG1				

Figure 2 Dot-blotting of protein extracts from human ileum and colon with anti-5-HT_{2B} antibody or control (mouse IgG1) antibody. Tissues are full thickness (whole), or sub-divided into mucosa and smooth muscle, and isolated *taenia coli* for colon. NA indicates not applicable to ileum. Data were obtained in tissues from a single donor.

Immunocytochemistry

In sections of human colon (from a single donor), 5-HT_{2B} receptor-like immunoreactivity (5-HT_{2B}R-ir) was observed in several cell types. In the muscularis externa, moderate 5-HT_{2B}R-ir was seen in the circular and longitudinal muscle layers, while moderate to strong staining was observed in putative myenteric nerve plexuses lying between the two muscle layers (Figure 3). Confirmation that 5-HT_{2B}R-ir was localized in the nerve plexus was obtained by labelling a neighbouring section of colon with a neuronal marker antibody, neurofilament 68 (Figure 4, upper and middle panel). The possibility that glial cells are also labelled can not be excluded. The control section, incubated with mouse IgG, showed no discernible staining (Figure 4 lower panel).

Pharmacology

Electrical stimulation (15 V, 1 ms pulse width, at sub-maximal frequency, for 10 s every 60 s) caused highly reproducible, transient, contractile responses of isolated preparations of human colon smooth muscle. The responses were inhibited by tetrodotoxin at a concentration of 1 or 3 μ M (inhibition of $88 \pm 12\%$ and $101 \pm 1\%$, $n=4$ and 6 respectively), or by atropine at a concentration of 1 or 10 μ M (inhibition of $84 \pm 7\%$ and $89 \pm 6\%$, $n=5$ and 6 respectively), indicating that they were neuronal in nature, and they involved (at least in part) cholinergic neurotransmission (data not shown).

Application of 5-HT produced a significant, concentration-dependent potentiation of the contractile response to electrical stimulation, with no significant effect on basal tone (Figures 5 and 6). The EC₅₀ for 5-HT was 8.2 ± 0.1 with a Hill slope of 1.3 ± 0.8 (both $n=49$). There was no evidence of

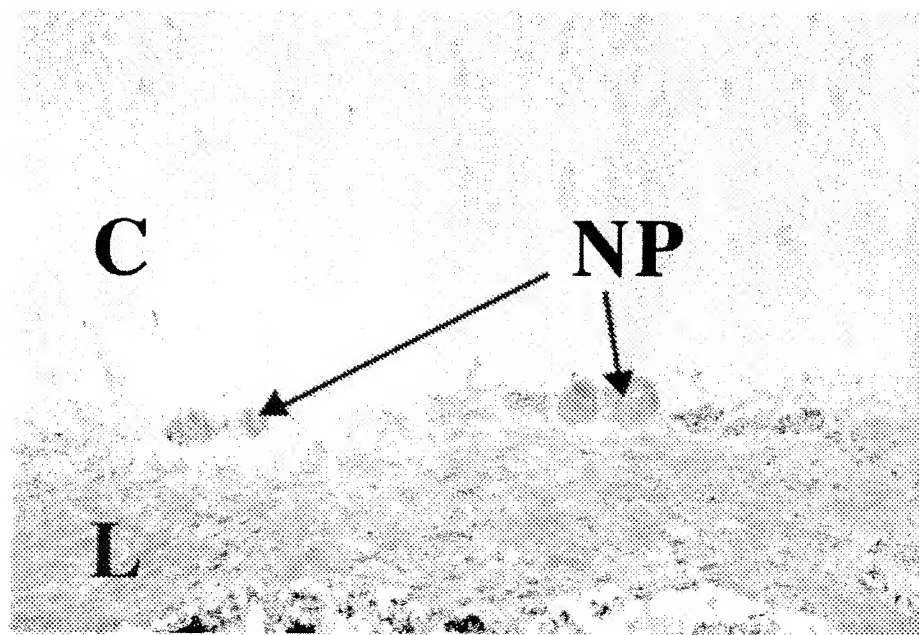


Figure 3 5-HT_{2B} receptor-like immunoreactivity in a paraffin-embedded section of human colon. In the muscularis externa, immunoreactivity was highest in the longitudinal muscle layer (L) and in the nerves of the myenteric nerve plexus (N), with lower expression in the circular muscle layer (C).

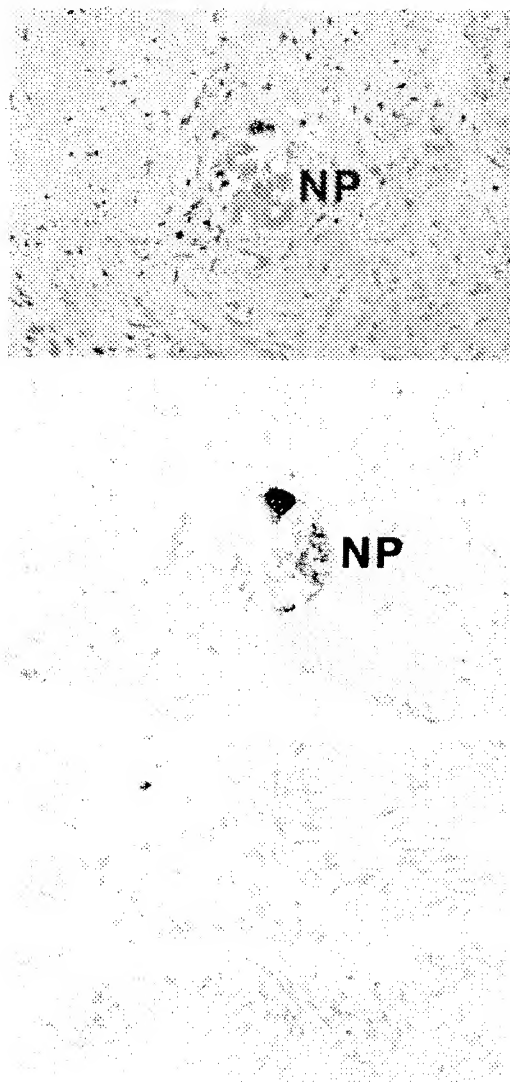


Figure 4 5-HT_{2B} receptor-immunoreactivity (5-HT_{2B}R-ir) and neurofilament-immunoreactivity (ir) in frozen sections of colon. 5-HT_{2B}R-ir in muscle and nerve plexus (NP, upper panel). Neurofilament-ir in a neighbouring section showing localization of the nerve plexus (middle panel), and control IgG₁ antibody (lower panel).

desensitization to increasing concentrations of 5-HT during the cumulative concentration-effect curve, although a small inhibitory effect of 5-HT was evident at higher concentrations (above 10^{-7} M), in some tissues. A range of 5-HT receptor agonists was also shown to cause potentiation of the contractile response. The order of agonist potency was α -Me-5-HT (5-HT₂ receptor agonist) > 5-HT (non-selective agonist) > 2-Me-5-HT (5-HT₃ receptor agonist) \geq 5-MeOT (5-HT₄ receptor agonist) > cisapride (5-HT₄ receptor agonist), which is consistent with a receptor of the 5-HT₂ family (Table 1). Application of a range of 5-HT receptor antagonists caused significant, rightward shifts of concentration-response curves to 5-HT, and the profile generated corresponds to the receptors involved being of the 5-HT_{2B} receptor class (Table 2). In particular, application of a highly potent and selective 5-HT_{2B} receptor antagonist, RS-127445 (Bonhaus *et al.*, 1999), caused a rightward displacement of the concentration-effect

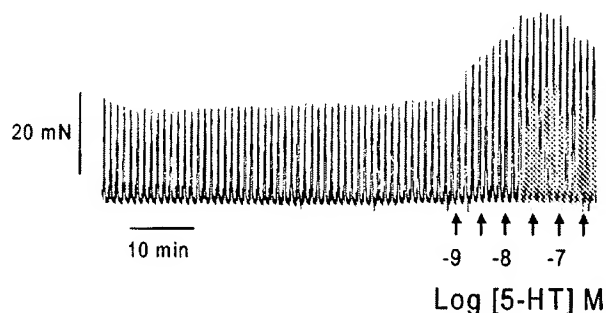


Figure 5 Typical contractile responses to 5-HT in electrically-stimulated human colon longitudinal smooth muscle. Figure shows the transient contractile response to EFS, and the potent, concentration-dependent potentiation of this neurally-mediated response by increasing concentrations of 5-HT (10^{-9} to $10^{-6.5}$ M in half log increments). At concentrations in excess of 10^{-7} M, 5-HT induces mild inhibition of electrically-stimulated contractions.

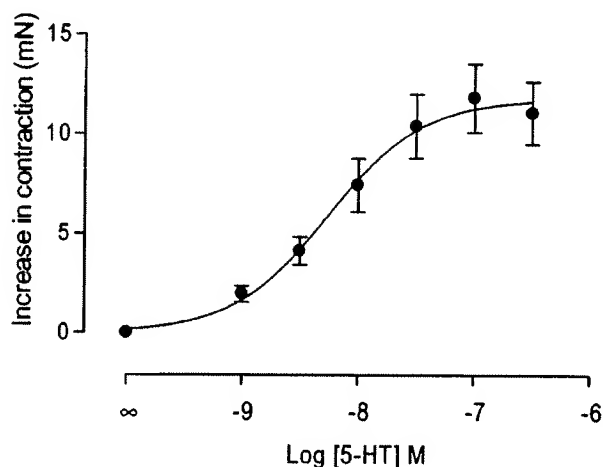


Figure 6 Effect of 5-HT on electrical field stimulation (EFS)-induced contractions of human colon longitudinal smooth muscle. Figure shows mean concentration-effect curve to 5-HT, with data expressed as increase in contractility (mN) over basal EFS-induced contractions. Data are given as mean \pm s.e. mean for $n=49$ donors, and have been fitted to the Hill equation according to a three parameter curve fit.

Table 1 Potencies of some 5-HT receptor agonists in human colon longitudinal muscle

Agonist	pEC_{50}
α -Me-5-HT	8.5 ± 0.1
5-HT	8.2 ± 0.1
2-Me-5-HT	7.0 ± 0.4
5-MeOT	6.8 ± 0.2
Cisapride	5.8 ± 0.3

Data are mean pEC_{50} values (\pm s.e. mean) in tissues taken from at least three donors. These agonists are not specific for a particular receptor sub-type, although α -Me-5-HT shows some selectivity for 5-HT₂ receptors over the other 5-HT receptor sub-types.

curve to 5-HT, with no significant alteration of the maximum response to 5-HT (Figure 7). Schild analysis generated a plot with slope not significantly different from unity (0.9 ± 0.2), yielding a pK_B of 9.4 ± 0.4 (Figure 8).

Table 2 Affinities of some 5-HT receptor antagonists against 5-HT-induced responses in human colon longitudinal muscle

Antagonist	Concentration range (nM)	pK _B
RS-127445	0.3–100	9.4 ± 0.4
SB 206553	10–100	8.5 ± 0.1
Rauwolscine	30–300	7.9 ± 0.2
Yohimbine	100–1000	7.5 ± 0.2
Methiothepin	100	7.5 ± 0.3*
Cisapride	30–1000	7.1 ± 0.1
SB 204741	100–10000	6.8 ± 0.2
SB 242084	3–1000	6.3 ± 0.2
Ketanserin	1000–10000	6.1 ± 0.2
Methysergide	1	NSA

Mean pK_B (*pA₂) estimates in human colon, where each antagonist was tested over at least three concentrations within the range stated, except methysergide and methiothepin which were each tested at a single concentration only. All data were obtained for $n=3-6$ donors. NSA indicates non-surmountable antagonism at the concentration tested. Of the antagonists tested, RS-127445 is the most selective 5-HT_{2B} receptor antagonist, displaying over 1000 fold selectivity over 5-HT_{2A} and 5-HT_{2C} receptors (Bonhaus *et al.*, 1999), whereas SB-204741 shows over 10 fold selectivity. The antagonist profile corresponds to a 5-HT_{2B} receptor.

Discussion

In the present study, we have investigated the role of 5-HT_{2B} receptors in controlling human colonic motility. The mRNA for this receptor is highly expressed throughout the human gastrointestinal tract, including colon. The protein for the receptor is localized within the smooth muscle of human colon, and in particular within the longitudinal smooth muscle and on the myenteric plexus, in accordance with a role in controlling colonic motility. In human colon longitudinal smooth muscle, we demonstrated that electrical stimulation of smooth muscle strips evoked a transient contractile response that could be inhibited by either atropine or tetrodotoxin, implicating the involvement of cholinergic nerves in the response. Exogenously applied 5-HT caused an increase in the magnitude of these neurally-mediated contractions, probably by either increasing the release of excitatory neurotransmitters such as acetylcholine, or by effects on the smooth muscle itself. The agonist profile of the response, and in particular the high potency of α -Me-5-HT, suggested that the response was mediated by a receptor of the 5-HT₂ family. Using a range of selective antagonists, the receptors were characterized as being of the 5-HT_{2B} sub-type. In particular, the highly potent and selective 5-HT_{2B} receptor antagonist, RS-127445, antagonized the response to 5-HT, with a pK_B in accordance with its reported affinity at the human 5-HT_{2B} receptor (Bonhaus *et al.*, 1999). Schild analysis yielded a plot with unit slope, indicating that the antagonism was competitive in nature, and that the response is likely to be mediated by a homogenous receptor population.

There are considerable differences in the nature of the receptor populations mediating the excitatory effects of 5-HT in human and animal colon. In the present study, we have demonstrated that in human colon the excitatory effects of 5-HT are mediated by 5-HT_{2B} receptors. In studies in laboratory animals, however, it has been shown that these

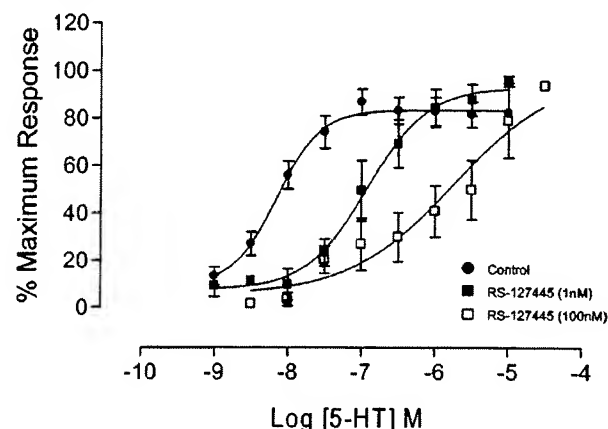


Figure 7 Antagonist effect of RS-127445 on the concentration-effect curves to 5-HT in human colon longitudinal smooth muscle. Figure shows mean concentration-effect curves to 5-HT in the absence and presence of RS-127445. Additional concentration-effect curves to 5-HT, in the presence of different concentrations of RS-127445 (see Figure 8), have been omitted for clarity. Data are expressed as percentage of the maximum response to 5-HT in the absence of antagonist, and are given as mean \pm s.e.mean for $n > 4$ donors.

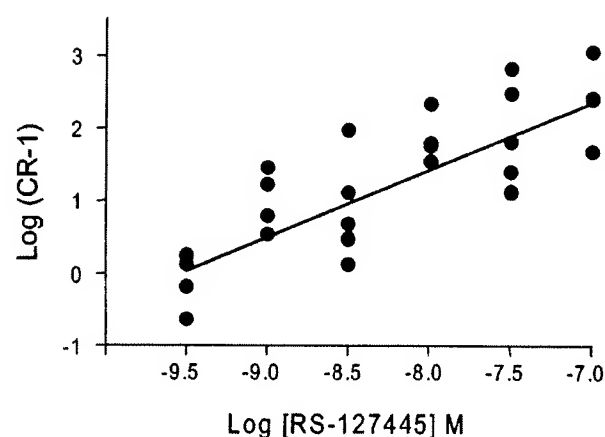


Figure 8 Schild plot of the antagonist effect of RS-127445 on responses to 5-HT in human colon longitudinal smooth muscle. The antagonism generated a pK_B of 9.5 ± 0.4 and slope of 0.9 ± 0.2 . Data are for $n > 4$ donors.

receptors do not play a significant role in the excitatory effects of 5-HT. In contrast, 5-HT₃ and/or 5-HT₄ receptors are responsible for 5-HT-induced excitatory effects in guinea-pig colon (Jin *et al.*, 1999), and 5-HT_{2A} and/or 5-HT₄ receptors have been proposed to be involved in canine colon (Prins *et al.*, 1997; 2000). In rat colon, contractions to 5-HT have been reported that are mediated either by 5-HT₁-like and putative 5-HT₃ receptors (Gelal & Guven, 1998), or by 5-HT₁ and 5-HT₄ receptor sub-types (Grider *et al.*, 1996). Although no selective antagonists of 5-HT₃ or 5-HT₄ receptors were tested in the present study, the fact that all the 5-HT_{2B} receptor antagonists tested inhibited 5-HT-induced effects, with potencies consistent with 5-HT_{2B} receptor antagonism, supports the involvement of a 5-HT_{2B} receptor. Of these antagonists, RS-127445 is known to be highly selective for 5-HT_{2B} receptors with at least 1000 fold selectivity over other 5-HT receptors (Bonhaus *et al.*, 1999).

Furthermore, the unit slope of the Schild plot for the antagonism of 5-HT by RS-127445 makes it extremely unlikely that more than one receptor sub-type is responsible for the effects of 5-HT. It is therefore clear that caution must be exercised when extrapolating from data obtained in animal studies, to predict properties of human tissues.

The precise mechanism by which 5-HT can potentiate neuronal responses in human colonic smooth muscle has yet to be determined. In animals, application of exogenous 5-HT has been shown to induce the release of acetylcholine from myenteric nerves (Yau *et al.*, 1990). Although our data are consistent with such a mechanism, there is as yet no direct evidence that increased neurotransmitter release is responsible for the effects of 5-HT in human colon. We have shown in the present study that 5-HT_{2B} receptors are localized both on colon smooth muscle, and on the nerves of the myenteric plexus. It is therefore possible that 5-HT may be exerting effects on the smooth muscle directly, instead of (or in addition to) increasing the release of excitatory neurotransmitters.

The present study investigated the effects of 5-HT on the longitudinal muscle layer that runs between the *taenia coli*. Previous studies have investigated 5-HT-induced inhibitory responses in the circular muscle of human colon, and excitatory effects of 5-HT on the *taenia coli* itself. Whilst

the precise roles of these different muscle layers in controlling human colonic motility has thus far not been elucidated, it is believed that colonic motility is a result of the co-ordinated contraction of both circular and longitudinal muscle layers. The present study has used inter-*taenia* longitudinal smooth muscle strips as a model for human colonic motility, although it is appreciated that contraction of this smooth muscle layer is not the only factor that contributes to colonic motility.

In summary, the results of the present study indicate that in human colon, the excitatory effects of 5-HT are mediated by 5-HT_{2B} receptors. This contrasts with findings from animal studies, where there is substantial evidence for a role for 5-HT₃ and/or 5-HT₄ receptors. Indeed, based on such data from animal studies, ligands acting at 5-HT₃ and/or 5-HT₄ receptors have been proposed (and developed) for the treatment of IBS, but their therapeutic efficacy remains to be fully established. In the light of the present data, it would therefore seem that a 5-HT_{2B} receptor antagonist approach may be beneficial in the treatment of IBS.

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5-HT2B receptor-mediated serotonin morphogenetic functions in mouse cranial neural crest and myocardial cells

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SUMMARY

During embryogenesis, serotonin has been reported to be involved in craniofacial and cardiovascular morphogenesis. The detailed molecular mechanisms underlying these functions, however remain unknown. From mouse and human species, we have recently reported the cloning of 5-HT2B receptors which share signal transduction pathways with other 5-HT2 receptor subtypes (5-HT2A and 5-HT2C). In addition to phospholipase C stimulation, it appears that these three subtypes of receptor transduce a common serotonin-induced mitogenic activity, which could be important for cell differentiation and proliferation. We have first investigated the expression of 5-HT2 receptor mRNAs in the mouse embryo. Interestingly, a peak of 5-HT2B receptor mRNA expression was detected 8-9 days postcoitum, whereas there was only low level 5-HT2A and no 5-HT2C receptor mRNA expression at this stage. Expression of this receptor was confirmed by binding assays using a 5-HT2-specific ligand which revealed a peak of binding to membrane preparations from 9 days postcoitum embryos. In addition, whole mount *in situ* hybridi-

sation and immunohistochemistry on similar stage embryos detected 5-HT2B expression in neural crest cells, heart myocardium and somites. The requirement for functional 5-HT2B receptors between 8 and 9 days postcoitum is supported by culture of embryos exposed to 5-HT2-specific ligands; 5-HT2B high-affinity antagonist such as ritanserin, induced morphological defects in the cephalic region, heart and neural tube. These antagonistic treatments interfere with cranial neural crest cell migration, induce their apoptosis, and are responsible for abnormal sarcomeric organisation of the subepicardial layer and for the absence of the trabecular cell layer in the ventricular myocardium. This report indicates for the first time that 5-HT2B receptors are actively mediating the action of serotonin on embryonic morphogenesis, probably by preventing the differentiation of cranial neural crest cells and myocardial precursor cells.

Key words: G protein-coupled receptors, whole embryo culture, apoptosis, neurulation, mouse

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is one of the well known monoamine neurotransmitters, mitogens, and hormones, which mediate a wide variety of physiological effect, including peripheral and central actions. The large variety of 5-HT functions is paralleled by the pharmacological complexity of 5-HT receptors which can be classified into different families depending on their signalling pathways. The family including 5-HT1 and 5-HT5 receptors interacts negatively with adenylyl cyclase; the 5-HT2 receptor family is coupled to the activation of phospholipase C (PLC); the family, including 5-HT4, 5-HT6 and 5-HT7 receptors, activates adenylyl cyclase, whereas the 5-HT3 receptor is a ligand gated ion channel.

Recently, we have cloned the 5-HT2B receptor cDNA from the mouse (Loric et al., 1992) and human (Choi et al., 1994). These receptors are functionally coupled to Gq and to the ras signalling pathway, and can be considered as a ligand

dependent oncogene acting on protein kinase C (PKC) and MAPKinase activation (Launay et al., 1996). The development of a subtype-specific antiserum allowed a precise mapping of the distribution of the mouse 5-HT2B receptors. In adult mice, the major sites of expression of 5-HT2B receptors are the gut and heart, and there is also detectable levels of expression in the brain and kidney (Choi and Maroteaux, 1996). Interestingly, the expression of 5-HT2B receptor mRNA is observed as early as 8 days postcoitum (d.p.c.) of mouse embryonic development (Loric et al., 1992). Additionally, the 5-HT2 receptor homologue in *Drosophila* is expressed during gastrulation (Colas et al., 1995), and the 5-HT2B receptors are also expressed during the serotonergic differentiation of the mouse teratocarcinoma derived IC11* cell lines (Loric et al., 1995; Kellermann et al., 1996).

Several line of evidence indicate that 5-HT possess developmental functions (Lauder, 1988; Lauder, 1993). For instance, it has been shown that 5-HT is present early in mammalian embryonic development and is probably maternally derived

(Yavarone et al., 1993a). Moreover in the mouse, the ability to take up 5-HT is detected at early embryonic stages (8.5 d.p.c., 10 somite pairs) in the heart myocardium (Shuey et al., 1993) and in the rhombencephalic neuroepithelium where it is restricted to rhombomeres 3 and 5 (Shuey et al., 1993). The physiological relevance of these findings is stressed by the data showing that embryos grown in the presence of high levels of 5-HT or 5-HT uptake blockers develop deficient head mesenchyme, hypoplastic mandibular arches and forebrain, open cranial neural folds and abnormal eyes. These malformations overlap those observed in whole embryo culture following exposure of mouse embryos to 13-*cis*-retinoic acid (Lauder, 1988; Lauder et al., 1988). 5-HT has also been shown to participate in rat craniofacial development (Van Cauteren et al., 1986) and to be involved in the formation and migration of the cranial neural crest (NC) cells (Moiseiwitsch and Lauder, 1995) as well as in chicken and mouse cardiovascular morphogenesis (Huether et al., 1992; Yavarone et al., 1993b).

Here we used molecular biology, pharmacology and whole embryo culture techniques to present evidence for involvement of 5-HT_{2B} receptors in mouse embryonic development at the neurulation stage.

MATERIALS AND METHODS

RT-PCR and binding assay

Total RNA from 8-13 d.p.c. mouse embryos was isolated using the guanidinium-thiocyanate method. Reverse transcriptase-PCR (RT-PCR) experiments were performed using specific oligonucleotides for mouse 5HT_{2A}, 2B, 2C, receptors and for the serotonin transporter (SERT) as follows:

5-HT_{2A}-AAGCCTCGAACTGGACAATTGATG/AAGATTTCA-GGAAGGCTTTGGTT

5-HT_{2B}-CAGAAGACATGTGATCACCTGATC/TGTAATCTTG-ATGAATGCAGTAGCC

5-HT_{2C}-GTTTGGCAGTTCGATATTCCTATA/GGCAATCTTCA-TGATGTAGTC

SERT-ACATCTGGCGTTTTCCCTACAT/TTTTGACTCCTTTC-CAGATG CT

To avoid the possible amplification of genomic DNA, oligonucleotides were designed in different exons. RT-PCR reaction was tested with different amounts of RNA (3, 4, 5 µg/50 µl reaction) to keep the reaction in the exponential phase for quantitative analysis of mRNA, and its specificity was verified by Southern blotting and hybridisation to a third primer.

Binding assays were performed with embryo membranes using [¹²⁵I]2,5-dimethoxy-4-iodophenyl-2-aminopropane ([¹²⁵I]DOI), alone or in presence of ketanserin, mesulergine or ritanserin (10⁻⁸ M) as described by Loric et al. (1992). Membrane extracts were also used for SERT quantification by binding experiments using [³H]paroxetine and for tryptophan hydroxylase (TPH) activity dosage by enzymatic assay according to published procedures (Buc-Caron et al., 1990).

Whole-mount in situ hybridisation, immunohistochemistry

For whole-mount in situ hybridisation, embryos were fixed with 4% paraformaldehyde followed by dehydration in serial dilutions of methanol and stored at -20°C in methanol until use. Hybridisation experiments were performed as described by Wilkinson and Dourish (1991), using whole 5-HT_{2B} cDNA sense or antisense probes. Embryos were fixed with 3% paraformaldehyde and then treated for whole-mount immunohistochemistry with an affinity-purified antiserum specific for 5-HT_{2B} receptors as described by Mark et al. (1993) and Choi and Maroteaux (1996).

Whole embryo culture

CD1 strain mouse embryos were cultured as described by Morriss-Kay (1993). Briefly embryos were dissected in cold Tyrode's solution, and then placed in 30 ml sterile glass bottles with 2.5 ml rat serum, 2.5 ml Tyrode's buffer, 0.1 ml penicillin/streptomycin. The bottles were filled with a mixture of 90% nitrogen, 5% oxygen, 5% carbon dioxide. Embryos of 8 d.p.c. (around 5 somite pairs) were incubated for 24 hours on a rotator wheel (30 revolutions per minute) at 37.5°C (Bavik et al., 1996). Drugs were dissolved in water or ethanol and then diluted at least 1000× (5 µl/5 ml) in culture medium. The morphological analysis is based on that described by New (1990). Experiments were performed at least 4 times with 4 or 5 treated embryos each.

Histological and electron microscopic analysis

Embryos were fixed by immersion in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) overnight at room temperature and washed in cacodylate buffer for further 30 minutes followed by postfixation with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at 4°C. Embryos were dehydrated through graded alcohol (50, 70, 90, 100%) for 30 minutes each and embedded in Epon 812. Semithin sections were cut at 1.5 µm and stained with toluidine blue, and histologically analysed by light microscopy. Ultrathin sections were cut at 70 nm and contrasted with uranyl acetate and lead citrate, and examined with a Philips 208 electron microscope. For scanning electron microscopy, embryos were fixed, dehydrated as above, dried with critical point-drying apparatus, and then mounted on aluminium stubs coated with palladium-gold using a cold sputter-coater and observed with a Philips XL-20 microscope.

Nile Blue sulphate staining

Nile Blue sulphate (NBS) dye staining of apoptotic cells was performed as described by Jeffs et al. (1992) except that phosphate-buffered saline was used as the incubation medium.

Embryo staging

The staging of the mouse embryos follows that described by Hogan et al. (1994) with the following criteria: 8 d.p.c. corresponds to 1-7 somite pairs; 8.5 d.p.c. to 8-12 somite pairs (embryo turning); 9 d.p.c. to 13-20 somite pairs; 9.5 d.p.c. to 20-30 somite pairs and 10 d.p.c. to 30-35 somite pairs.

RESULTS

5-HT_{2B} receptors are highly expressed at 8 to 9 d.p.c.

Previous studies have indicated that different 5-HT₂ receptors are expressed early during rodent embryonic development (Loric et al., 1992; Hellendall et al., 1993). We have refined these data by performing semi-quantitative RT-PCR on RNA extracts from staged embryos using a set of amplimers specific for the 5-HT_{2A}, 2B and 2C receptors. Representative results of such experiments are shown in Fig. 1A,B. We observed a strong expression of 5-HT_{2B} mRNA at 8 d.p.c. followed by a sharp decreased by 10 d.p.c. In contrast, low levels of 5-HT_{2A} mRNA were detected at 8 d.p.c. and rose slowly. The expression of 5-HT_{2C} mRNA appeared only at 13 d.p.c. in parallel with a second wave of 5-HT_{2B} mRNA expression. Interestingly, elevated levels of SERT mRNA and protein (as [³H]paroxetine binding sites) were detected from 8 to 10 d.p.c. (Table 1; Fig. 1A). Binding experiments performed on protein extracts from similarly

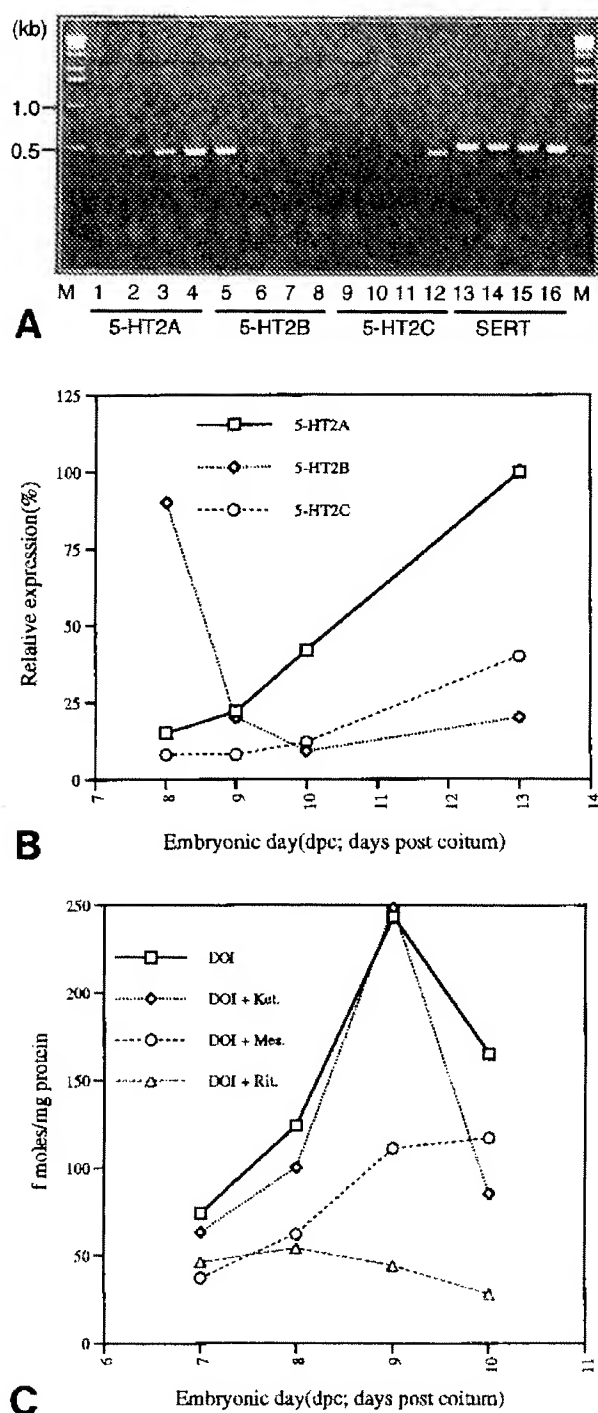


Fig. 1. 5-HT2B receptors are expressed at 8 and 9 days postcoitum. (A) mRNA expression levels of 5-HT2 receptors and SERT. The expression pattern of 3 different subtypes of 5-HT2 receptors and SERT during mouse embryogenesis (8, 9, 10, 13 d.p.c.) have been analysed by quantitative RT-PCR: 8 d.p.c., lane 1, 5, 9, 13; 9 d.p.c., lane 2, 6, 10, 14; 10 d.p.c., lane 3, 7, 11, 15; 13 d.p.c., lane 4, 8, 12, 16. (B) Densitogram analysis of quantitative RT-PCR, 100% represents the highest level of expression (5-HT2A at 13 d.p.c.). (C) Specific binding of [125 I]DOI to 5-HT2 receptors alone (DOI) or in the presence of 10^{-8} M of ketanserin (Ket), mesulergine (Mes) or ritanserin (Rit).

staged embryos, using [125 I]DOI, a specific 5-HT2 radioligand, revealed a peak of specific binding at 9 d.p.c. (Fig. 1C). The specific binding of [125 I]DOI was completely blocked by ritanserin, partially reduced by mesulergine and totally insensitive to ketanserin. Given the respective affinity of these drugs for the different 5-HT2 receptors (Table 2), these results indicate that the peak of [125 I]DOI binding detected at 9 d.p.c. is mainly due to the expression of 5-HT2B receptors.

The 5-HT2B receptor protein is strongly expressed in brain and heart at the neurulation stage

Further refinement of the 5-HT2B receptor expression was obtained by studying its mRNA distribution by whole mount in situ hybridisation. Strong expression of the 5-HT2B receptor mRNA was detected at 8 d.p.c. in the neural fold, neural tube and heart primordia (Fig. 2A). This expression persisted in the cephalic region, neural tube and heart at 9 d.p.c. (Fig. 2B). This mRNA expression was confirmed by immunostaining whole-mount embryos using a 5-HT2B C-terminal-specific antiserum (Choi and Maroteaux, 1996): 5-HT2B receptor protein appears to be expressed in the somites, neural tube and heart region at 8.5 d.p.c. (Fig. 3B,D), and persisted in the same regions with addition of the otic and optic vesicles, and pharyngeal arches at 9.5 d.p.c. (Fig. 3F,H).

5-HT2B receptor blocking between 8 and 9 d.p.c. induces embryonic defects

Early embryonic expression of 5-HT2B receptors raised the possibility that these receptors are involved in 5-HT-dependent morphogenetic processes. Therefore, we initiated a series of experiments to test this hypothesis by performing whole embryo culture. Embryos at about the 5 somite-pairs stage were incubated for 24 hours in medium containing 1:1 rat serum/Tyroses salt solution and various serotonergic drugs. Normal rat serum contains nearly micromolar amount of 5-HT ($0.5\text{--}1\text{ }\mu\text{M}$) and dialysed serum cannot support embryonic development in our experimental conditions, even supplemented by $1\text{ }\mu\text{M}$ 5-HT. Therefore, in order to block the action of 5-HT2B receptors, we selected specific antagonists of the 5-HT2 subtypes of receptors, ritanserin, methysergide, mesulergine, ketanserin and mianserin, for their differential affinity for these receptors (Table 2). No defects were detected in embryos incubated in normal serum, whereas treatment with antagonist induced several reproducible embryonic defects which were characterised by optical microscopy (Fig. 4A-C), and by scanning electron microscopy (Fig. 4D-F). As shown in (Fig. 4B,E) ritanserin-

Table 1. Serotonergic properties of embryonic extracts

Embryonic stage (d.p.c.)	Paroxetine binding sites (fmol/mg prot.)	Tryptophan hydroxylase (fmol/hours/mg prot.)
8	89.6	<0.3
9	88.7	<0.3
10	86.8	<0.3
13	61.7	1.4

Extracts of embryos at various stages were analysed for the presence of SERT sites using [^3H]paroxetine as the labelled compound for binding experiments on membrane fractions, and for TPH enzymatic activity dosage, according to published procedures (see Buc-Caron et al., 1990).

Table 2. Binding affinity of several antagonists to 5-HT₂ receptors

Receptors	Antagonist				
	Ritanserin	Methysergide	Mesulergine	Ketanserin	Mianserin
5-HT _{2A}	9.28±0.12	8.58±0.13	8.21±0.07	8.74±0.21	8.09±0.18
5-HT _{2B}	8.39±0.17	7.86±0.13	7.67±0.22	6.74±0.15	6.42±0.10
5-HT _{2C}	8.68±0.08	8.25±0.10	8.77±0.18	6.90±0.15	7.91±0.24

Data correspond to pK_i values (−logK_i) obtained by competition of [¹²⁵I]DOI binding with various compounds on membranes from mouse L cells transfected with mouse 5-HT_{2A}, 5-HT_{2B} or 5-HT_{2C} receptor cDNA. Values represent K_i±s.e.m. of experiments performed three times in triplicate.

treated embryos show a strong growth retardation as compared to controls (Fig. 4A,D). The yolk sac circulation was also impaired as indicated by the formation of few blood islands. Moreover, the cephalic region showed apparent defects in flexure, and the forebrain, hindbrain and the first pharyngeal arch developed abnormally. In addition, the epicardial layer of the ventricular wall was swollen. Defects in embryonic turning, in somite number and shape, and in neural tube shape and closure were also frequently observed after ritanserin treatment (Fig. 4B,E; Table 3). Ritanserin induced these defects in all treated embryos at 1 µM concentration (Table 3), whereas at 100 nM ritanserin nearly 20% of the embryos were already affected (not shown). Defects could be visually detected 6 hours after the beginning of treatment. Simultaneous addition of agonist with ritanserin, 5-HT (Fig. 5; Table 3) or the highly specific 5-HT_{2B} agonist N-acetyl-5-HT (NAS) (Colas et al., 1997), prevented the onset of these defects in almost 50% of the treated embryos (Table 3). Interestingly, among the other 5-HT antagonists tested (Table 3) only those having a high affinity for 5-HT_{2B} receptors (Table 2), gave a similar phenotype to that of ritanserin. In contrast, the antagonists mianserin (not shown) and ketanserin which have a lower affinity for 5-HT_{2B} than for 5-HT_{2A} or 5-HT_{2C} receptors (Table 2), gave a milder phenotype even at a concentration of 10 µM (Table 3; Fig. 4C,F), pointing out a 5-HT_{2B}-mediated action.

5-HT_{2B} receptor antagonists block cranial NC cell migration and/or differentiation

The defects induced by 5-HT antagonists were further char-

acterised by light and transmission electron microscopy (TEM) on thin sections of these embryos. Toluidine blue stained sections showed that ritanserin-treated embryos have hypoplastic pharyngeal arches and an irregular neural tube with dilated blood vessels (Fig. 6B-H). It is important to point out that the reduced size of pharyngeal arches is indicative of impairment of NC cell migration and/or proliferation. The NC cells in the ritanserin-treated embryos, seemed not to migrate properly and remained in a more dorsal aspect of the cephalic region (Fig. 6F) than in the control embryos (Fig. 6E). Fig. 7 shows higher magnification of the neural crest region of these sections. Although dividing cells can be seen in the epithelium, densely stained pyknotic nuclei, which are indicative of dying cells, were specifically observed in NC cells of the first pharyngeal arch and in the neuroepithelium near the optic stalk. Several characteristics of apoptosis were identified, cells presenting electron dense nuclei with condensed chromatin, dark cytoplasm and fragmented cells, whereas no necrotic cells could be seen after ritanserin treatment (Fig. 7B-D). Counting the number of apoptotic NC cells on these sections gave an average of 52% apoptotic cells in ritanserin-treated embryos whereas only 6% were observed in vehicle-treated embryos. In order to confirm the apoptotic nature of these cells we stained embryos with the NBS dye which has been frequently used to visualise patterns of cell death during embryogenesis (Lumsden et al., 1991; Jeffs et al., 1992). The cephalic region of the ritanserin-treated embryos show extensive NBS staining over the pharyngeal arch 1, forebrain region and optic vesicle (Fig. 8B,D) in addition to the staining observed in control embryos over the rhombomeres 3 and 5 (Fig. 8A,C) already described (Graham

Table 3. Summary of whole embryo culture

Treatment	Concentration of drug (µM)	Number of tested embryos	Yolk sac circulation	Morphological defects (%)			
				Brain	Heart	Neural tube	Growth
Vehicle	none	20	—	0 (0)	0 (0)	0 (0)	0 (0)
Ritanserin	0.5	20	++	16 (80)	18 (90)	15 (75)	11 (55)
	1.0	20	++	20 (100)	20 (100)	18 (90)	18 (90)
Methysergide	10	16	+	9 (56)*	9 (56)	6 (37)	5 (31)
Ketanserin	10	20	—	18 (90)†	2 (10)	2 (10)	2 (10)
Ritanserin +5-HT	0.5	18	++	8 (44)	5 (27)	4 (22)	3 (16)
Ritanserin +NAS	0.5	16	+	5 (31)	7 (43)	5 (31)	4 (25)
	10						

Cultures of CD1 strain mice embryos were started at 8 d.p.c. (4-9 somite pairs) and incubated for 24 hours in 5 ml culture medium (50% rat serum, 50% Tyrode's saline) containing about 0.5-1 mM endogenous 5-HT. The number of treated embryos represents at least four independent experiments where 4-5 embryos have been treated at a time. The morphological analysis is based on that described by New (1990). The yolk sac observation corresponds to blood island formation after treatment, — not detected, + occasional blood island, ++ dispersed but reproducible blood island. The brain defects are mainly microcephaly; *defects of forebrain, 3 (19%) and hindbrain, 6 (37%) †defects in hindbrain only. The heart defects are essentially distended pericardium with defects in chamber formations. The neural tube defects correspond to irregular and often kinked neural tube. Finally, the growth defect is reduced size of the embryos.

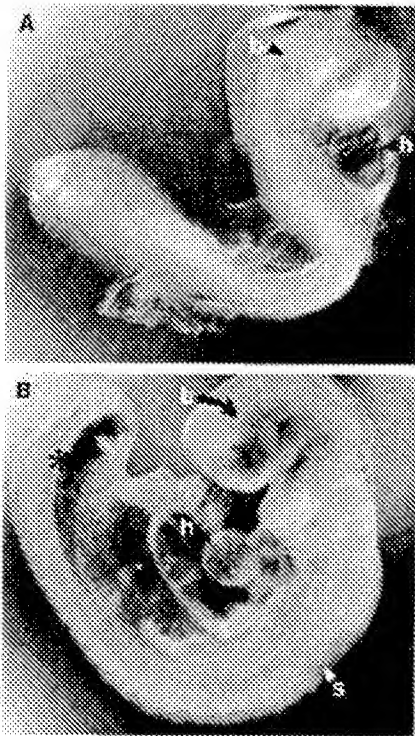


Fig. 2. 5-HT_{2B} receptor mRNA is expressed in brain and heart region. Whole-mount in situ hybridisations were performed using digoxigenin-labelled 5-HT_{2B} antisense RNA and show that 5-HT_{2B} receptors expression is mainly localised in brain and heart regions. (A) 8 d.p.c. embryo. Scale bar, 50 μ m. (B) 9 d.p.c. embryo. Scale bar, 100 μ m. b, cephalic neural plate region; h, heart region; s, somites; *, labelling of the caudal region including hindgut which was sometime observed with the sense probe and therefore may be non-specific.

et al., 1993, 1994; Lumsden and Graham, 1996). Furthermore, in treated embryos, the kinked neural tube and somites have more NBS-labelled cells than control embryos (Fig. 8C,D).

5-HT_{2B} receptor antagonist induces heart defects

Similar studies of the cardiac region were performed (Fig. 9). In ritanserin-treated embryos the heart structure was disorganised (Fig. 9B,D). In particular, the bulboventricular groove was not well defined and the swollen atrioventricular canal showed reticulocyte accumulation indicating an inefficient circulation (Fig. 9B). The subepicardial layer was thin and the cardiac trabecular cells were absent in the ventricle. Elongated cells, however, were present in this layer which normally contains myocardial trabecular stem cells (Fig. 9D). TEM analysis of thin sections of ritanserin-treated embryos (Fig. 9E,F) indicated that this compound induces abnormal differentiation of myofilament sarcomeres in the subepicardial layer. These anomalies were not detected in vehicle-treated embryos at the same stage. This results suggest either a modification of the differentiation program of the myocardial stem cells and/or a deficient migration of the precursors of the trabecular cells.

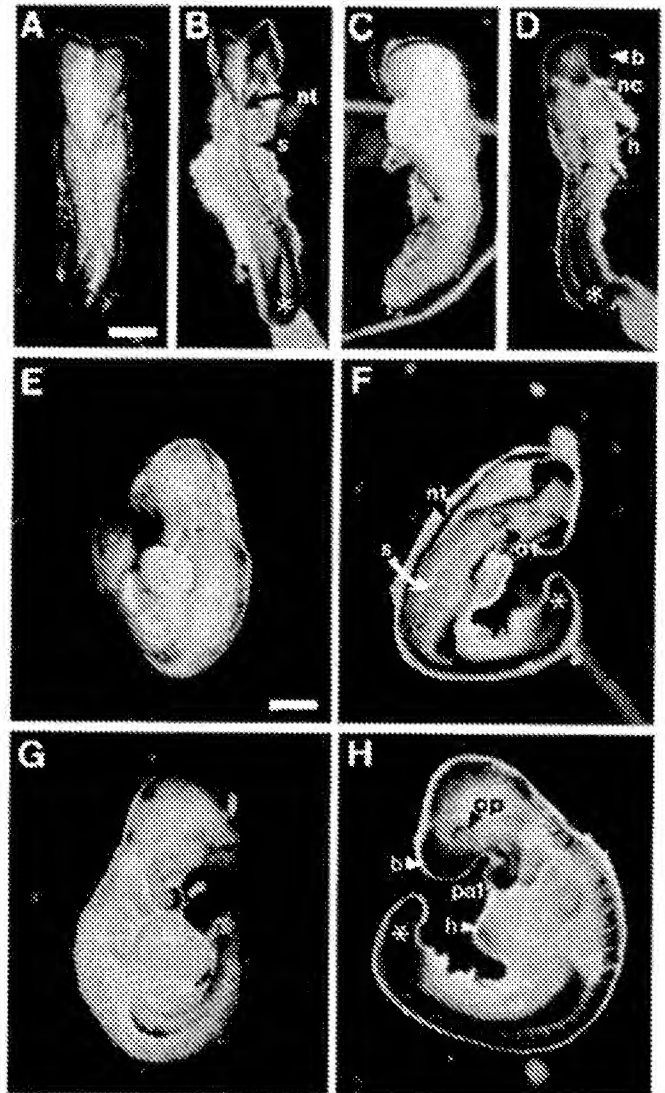


Fig. 3. 5-HT_{2B} receptor protein is expressed in forebrain, heart, neural tube and somites at 8.5 and 9.5 d.p.c. Whole-mount immunohistochemical localisation using an affinity purified antibody directed to the C-terminal peptide sequence of the 5-HT_{2B} receptor. B,D,F,H reveal expression of the receptor over the neural tube, pharyngeal arch 1 and heart. (A-D) 8.5 d.p.c. embryos. (A,C) Control embryos after incubation in presence of the 5-HT_{2B} antiserum with an excess of the immunising peptide. (E-H) 9.5 d.p.c. embryos. (E,G) Control embryos. Scale bar, 200 μ m. nt, neural tube; s, somites; b, forebrain region; h, heart; nc, neural crest cells; ot, otic vesicle; op, optic vesicle; pa1, first pharyngeal arch; *, staining of caudal region sometime revealed in the control embryos and therefore may be non-specific.

DISCUSSION

The presence of 5-HT has been detected during early mammalian embryonic development (Lauder et al., 1988). We observed that the limiting enzyme in 5-HT biosynthesis, tryptophan hydroxylase (TPH) cannot be detected in embryos between 8 and 10 d.p.c. (Table 1), substantiating its suspected maternal origin (Yavarone et al., 1993a). Similarly, we detected

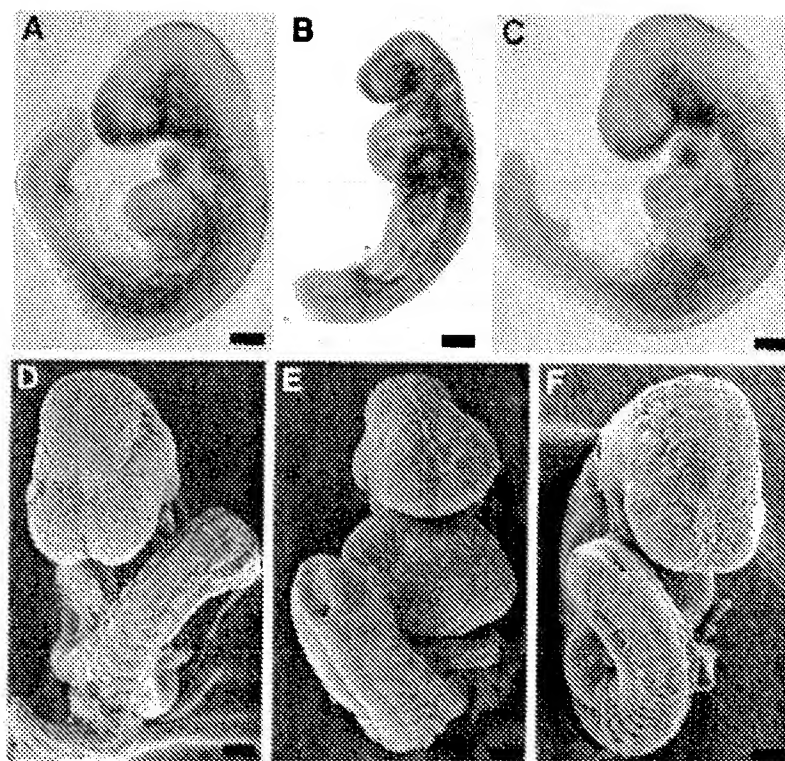


Fig. 4. Morphological analysis of whole embryo culture using light and scanning electron microscopy. CD1 strain mouse embryos were cultured with different 5-HT₂-specific antagonists from 8 to 9 d.p.c. (5 to 20 somite pairs) for 24 hours. (A,D) Control embryos. (B,E) Ritanserin (1 μ M) treated embryos showing morphological defects: the brain, the pharyngeal arches and the somites are not properly developed, the heart pericardium is swollen and the neural tube is not closed. (C,F) Ketanserin (10 μ M) treated embryos show a nearly normal development except over the hindbrain region. Scales bar, (A-C) 145 μ m; (D,F) 130 μ m; (E) 100 μ m.

the presence of binding sites at 8-10 d.p.c. for the SERT-specific ligand [³H]paroxetine (Table 1), corroborating the 5-HT uptake activity previously described at the 10 somite-pairs stage (8.5 d.p.c.; Lauder et al., 1988) in the neuroepithelium and myocardium (Shuey et al., 1993). Furthermore, 5-HT has been shown to affect rat and mouse craniofacial (Van Cauteren et al., 1986; Shuey et al., 1993) and chicken and mouse cardiovascular morphogenesis (Huether et al., 1992; Yavarone et al., 1993b). Some of these effects have been reported to be mediated by 5-HT₂-like receptors (Van Cauteren et al., 1986; Huether et al., 1992).

We describe here, an extensive identification of 5-HT receptors present at the neurulation stage of early mouse embryogenesis. We have focused on the 5-HT₂ receptor family since the mitogenic activity of 5-HT has been linked to 5-HT₂ receptor-dependent stimulation of PLC/PKC. (i) 5-HT has mitogenic effects on NIH3T3 fibroblasts expressing a high density of 5-HT_{2A} or 5-HT_{2C} receptors (Julius et al., 1989), as well as on LMTK⁻ cells expressing 5-HT_{2B} receptors, which in response form foci and induce tumours in nude mice (Launay et al., 1996). (ii) We have shown that, in these later cells, 5-HT_{2B} receptors are functionally coupled to IP₃ stimulation via the heterotrimeric α subunit of G_q and to the ras signalling pathway via the beta-gamma subunit of the same protein. These trigger the effects of 5-HT_{2B} selective agonists on PKC and MAPKinase activity in these cells (Launay et al., 1996). (iii) 5-HT acts on the serotonergic differentiation of the teratocarcinoma-derived cell line 1C11* which expresses 5-HT_{2B} receptors before 5-HT_{2A} receptors and regulates the final phenotype of these cells (unpublished data; Loric et al., 1995; Kellermann et al., 1996). These overall properties strongly suggest that 5-HT_{2B} receptors mediate some trophic functions of 5-HT.

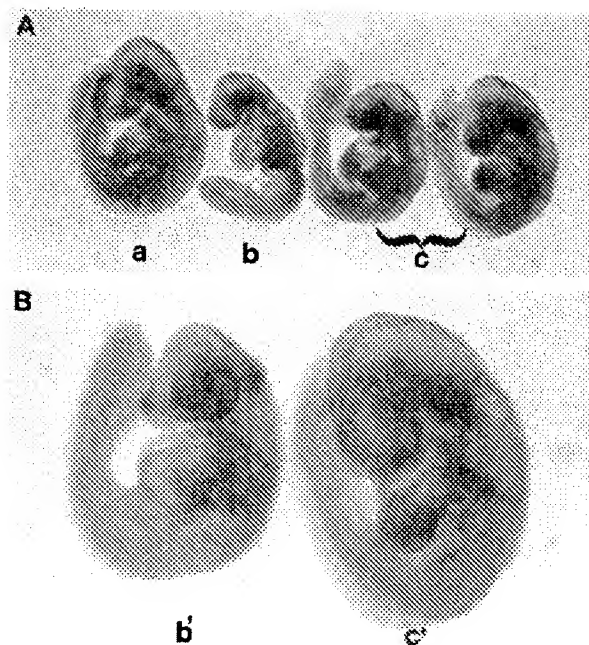
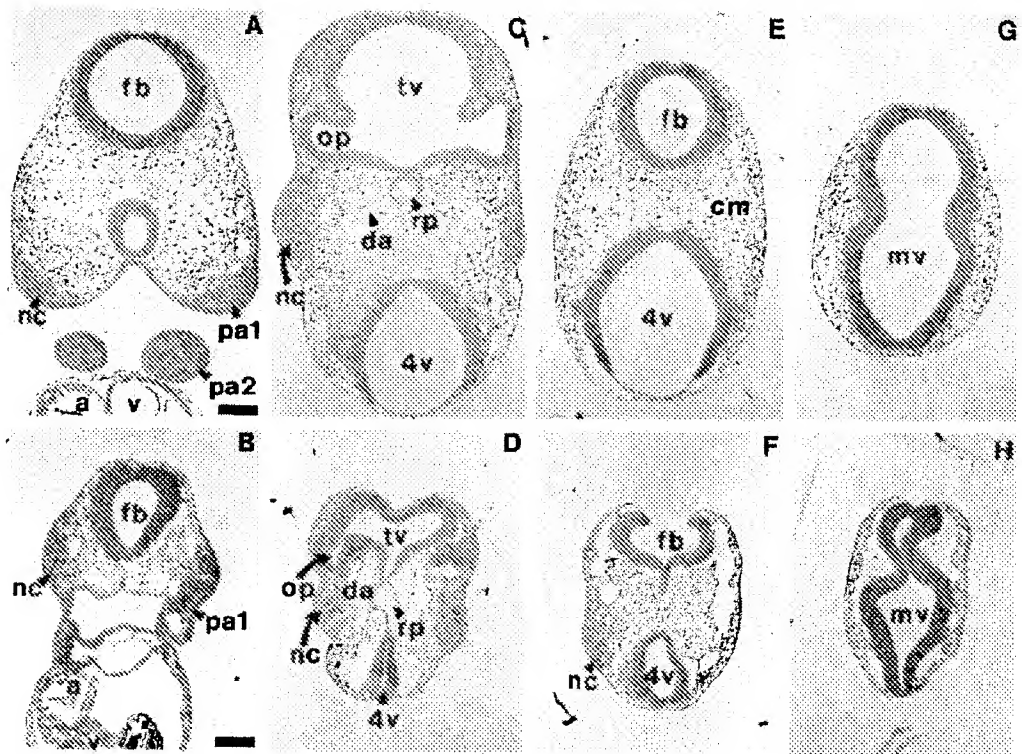


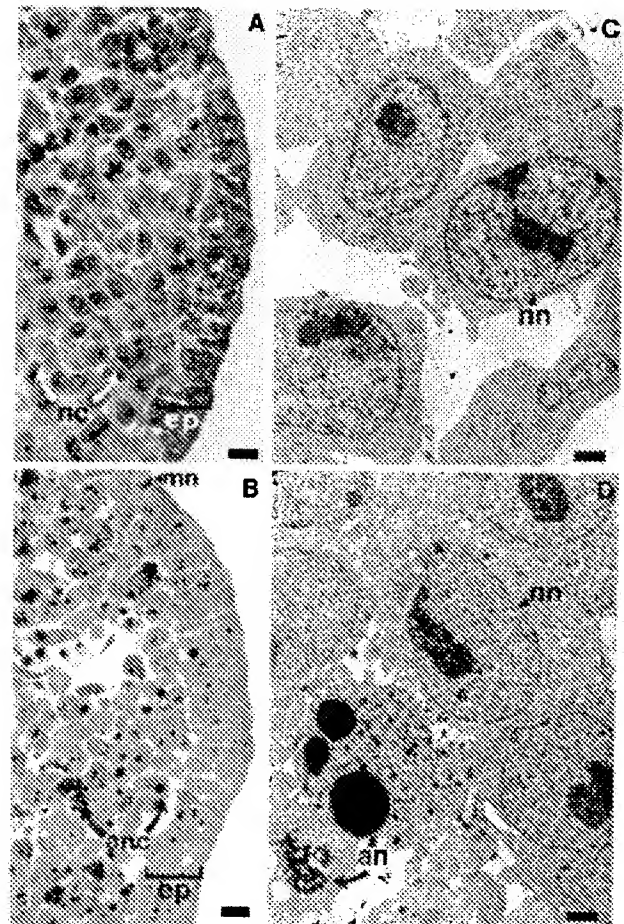
Fig. 5. Ritanserin-induced morphological defects can be prevented by addition of 5-HT. (A) Comparison of the different phenotypes: a, control embryo; b, ritanserin (0.5 μ M) treated embryo showing morphological defects in cephalic and heart region; c, addition of 5-HT (10 μ M) to ritanserin (0.5 μ M) treated embryo showing partial morphological improvement. (B) Comparison between ritanserin and 5-HT plus ritanserin treated embryos: b', c' are the enlargement of b, c and the 5-HT plus ritanserin-treated embryos present a phenotype close to the untreated embryos.

Fig. 6. Histological analysis of the cephalic region of cultured embryos. Transverse thin sections of control embryos (A,C,E,G) and of ritanserin treated embryos (B,D,F,H) have been performed after fixation and embedding in Epon. The treated embryos shows irregular organisation of the neural tube which is not completely closed (F,H). The fourth ventricle is reduced and blood vessels are dilated; NC cells are observed higher up than in the control (F) and the pharyngeal arches are not well formed (B). The magnification is the same for all the sections: scale bars, (A,B) 150 μ m. a, atrium; cm, cephalic mesenchyme; da, dorsal aorta; fb, forebrain; mv, mesencephalic vesicle; nc, neural crest; op, optic vesicle; pa, pharyngeal arch; rp, Rathke pouch; tv, telencephalic vesicle; v, ventricular chamber; 4v, fourth ventricle.



In the present study we have observed both by mRNA analysis (Fig. 1A,B) and receptor protein binding (Fig. 1C), that the 5-HT₂B subtype is the major 5-HT₂ receptor expressed between 8 and 9 d.p.c.. Whole-mount in situ hybridisation (Fig. 2) and immunohistochemistry experiments (Fig. 3) reveal expression of this receptor on the neural tube, cranial neural crest cells, on somites, and in the heart tube at 8.5 d.p.c. (Fig. 3B,D). At 9.5 d.p.c., the forebrain region, the pharyngeal arch 1 and the otic and optic vesicles are also positively labelled (Fig. 3F,H). At a later stage of development, 11.5 d.p.c., the cardiac staining is restricted to the trabecular cells of the myocardium (not shown). Interestingly, the 5-HT₂B staining at 8.5 d.p.c. is observed in the region of migratory NC cells (Fig. 3D), and the staining observed at 9.5 d.p.c. over the pharyngeal arch is suggestive of that of the cranial NC cells after migration. The 5-HT₂B receptor staining over the somites, neural tube and cranial

Fig. 7. Histological and electron microscopical analysis of the NC region of cultured embryos. In control embryos (A, C) the NC cells present a normal morphology. In the ritanserin (1 μ M) treated embryos (B,D), the morphology of NC cells is modified, with several fragmented nuclei and nuclei with condensed chromatin whereas mitotic nuclei are visible in the epithelium. At the EM level, several characteristics of apoptosis can be identified, including the appearance of electron dense chromatin masses, convolution of nuclear membrane, cell shrinkage and darkening of the cytoplasm. Apoptotic bodies containing intact organelles and fragmented chromatin can be observed. Some cell fragments are phagocytosed by macrophages or neighbouring cells. Scale bars, (A,B) 10 μ m; (C,D) 1 μ m. an, apoptotic nucleus; anc, apoptotic NC cells; ep, epithelium; mn, mitotic nuclei; nc, neural crest; nn, normal nucleus.



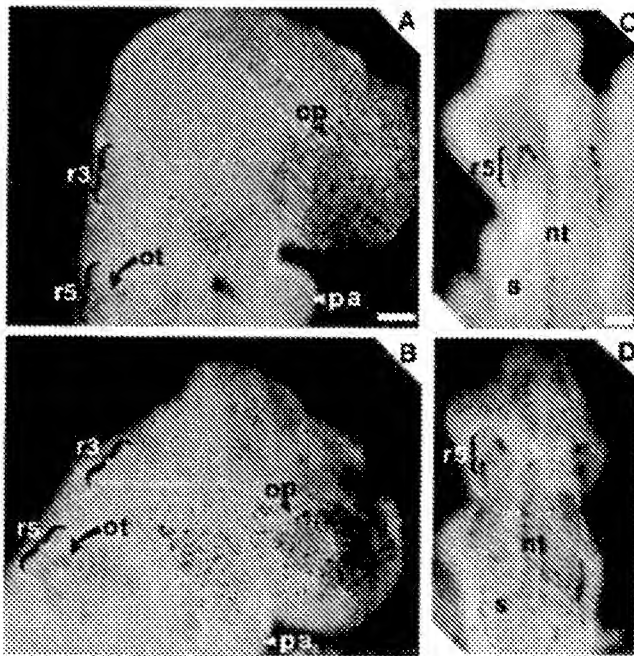


Fig. 8. Cephalic region of embryos labelled with NBS dye. Control embryos (A,C) and ritanserin (1 μ M) treated embryos (B,D) were stained using NBS dye, a procedure that has been used to visualise apoptotic cells during embryogenesis (Lumsden et al., 1991; Jeffs et al., 1992). The known regions of apoptotic NC cells are visualised at the level of rhombomeres 3 and 5 in both treated and control embryos whereas in the ritanserin-treated embryos, extensive labelling appears over the pharyngeal arch, in the forebrain region (B), and over the neural tube and somites (D). The magnification is the same in A and B, and in C and D, respectively, and the scale bars represent 160 μ m. nt, neural tube; op, optic vesicle; ot, otic vesicle; pa, pharyngeal arch 1; r3, r5, rhombomeres 3 and 5; s, somites.

neural crest is overlapping with that of the product of the paired box gene *Pax-3* (Goulding et al., 1993; Pourquié et al., 1995). This transcription factor is responsible for the *spotch* phenotype in mice and the Waardenburg syndrome in human (Wehr and Gruss, 1996) which primarily affect neural crest cell derivatives and its gene has a similar localisation to the 5-HT_{2B} receptor gene on chromosome 2q36 (LeConiat et al., 1996).

5-HT_{2B}-specific antagonist treatments induce developmental defects

The treatment of embryos with high affinity 5-HT_{2B} receptor antagonist induce a highly reproducible phenotype which includes strong growth retardation (Fig. 4B,E), abnormal flexure of the cephalic region, underdeveloped forebrain and hindbrain, small pharyngeal arches and distended epicardial layer (Figs 6, 8). Defects in embryo turning, in somite number and shape, and in neural tube shape and closure are also frequently observed after these treatments (Table 3). This phenotype, including small brain and pericardial oedema, is very similar to that observed in the mouse mutated for the *fused* gene (Perry III et al., 1995),

and in the knock-out mice phenotype of Ras GTPase-activating protein (Henkemeyer et al., 1995) and of the neurofibromatosis type-1 gene which is a regulator of the *ras* signal transduction pathway (Brannan et al., 1994). Finally, it is worth noting that a human pathological condition showing similar embryonic defects to those we observed, has been described in the embryos of phenylketonuria mothers (Lenke and Levy, 1980), and seems to be associated with low levels of blood 5-HT (Roux et al., 1995).

All the affected areas of the embryos express high levels of 5-HT_{2B} receptors (Figs 2, 3). In addition, the effects induced by ritanserin or mesulergine seem to be specific. First, morphological modifications induced by antagonist treatment are dose- (Table 3) and time-dependent, and can be partially prevented by simultaneous treatment with NAS, a highly selective 5-HT_{2B} receptor agonist or with 5-HT (Fig. 5; Table 3). Second, the yolk sac circulation impairment is not directly involved since nearly normal embryos were associated with blood island formation in ritanserin-plus-5-HT treatment (Table 3). Third, a general toxic effects of these compounds can be excluded since the defects are tissue-specific and, despite the presence on sections of apoptotic cells, dividing cells can still be observed (see Fig. 7B). Finally, the non-migrating NC cells undergo cell death with typical apoptotic but not necrotic morphology (Fig. 7D). The possibility that ritanserin acts at other 5-HT receptor subtypes cannot be completely excluded but is very unlikely since these compounds are typical 5-HT₂-specific ligands (Hoyer et al., 1994); the only known serotonin receptors having fairly high affinity for ritanserin are the rat 5-HT₆ and 5-HT₇ subtypes but their affinity for ritanserin is at least 10 times lower than that of mouse 5-HT_{2B} receptors (Boess and Martin, 1994).

5-HT_{2B} specific antagonists trigger differentiation of the heart myocardium

The 5-HT action on early embryonic development includes head mesenchyme and pharyngeal arch formation, neural tube closure, eye and heart development, and is partially overlapping with retinoic acid teratology (Lauder, 1988). The ritanserin treatment of embryos induced a modification of the differentiation program of the myocardial stem cells and/or a deficient migration of the precursors of the trabecular cells (Fig. 9). The absence of trabeculation in the myocardium is similar to that reported in neuregulins knock-out mice (for review see Lemke, 1996), and in RXR α mutated animals (Kastner et al., 1994). In both cases, the thickness of the ventricular wall is markedly decreased. The retinoic acid-induced transcription factor AP-2, when knocked-out, also gives a similar cardiac phenotype (Shorle et al., 1996; Zhang et al., 1996). Since the promoter region of the 5-HT_{2B} receptor contains consensus binding sequence for retinoic acid receptor and AP-2 (unpublished), we propose that the 5-HT_{2B} receptors are interacting with these transcription factors.

5-HT_{2B} specific antagonists activate apoptosis in cranial NC cells

In mouse, the NC cells emerge by epitheliomesenchymal transformation (Newgreen and Minichiello, 1995), from

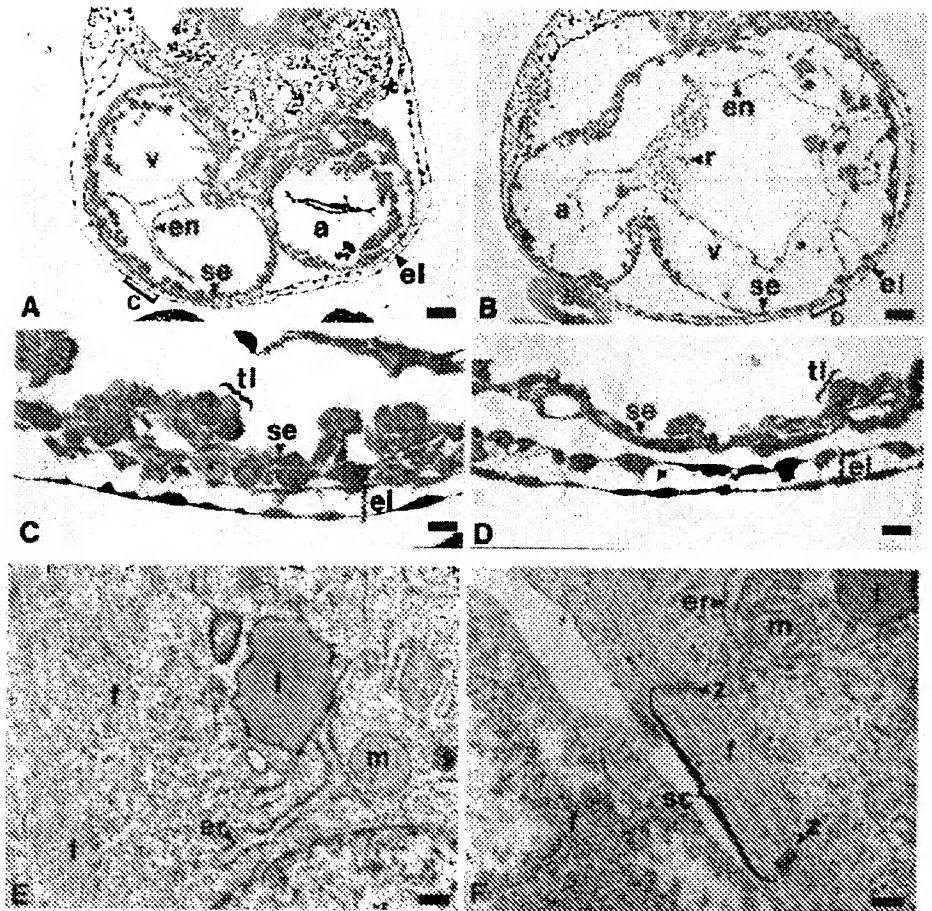


Fig. 9. Histological and electron microscopical analysis of the heart region of cultured embryos. Control embryos show a normal subepicardial layer with round cells (A,C,E), whereas ritanserin (1 μ M) treated embryos (B,D,F) have inflated hearts with defects of the trabecular layer and a distended endocardium with accumulation of reticulocytes. The subepicardial layer contains elongated cells presenting myofilaments with sarcomeric organisation (F) which is not yet formed in the control (E). Scale bars, (A,B) 50 μ m; (C,D) 10 μ m; (E, F) 0.14 μ m. a, atrial chamber; el, epicardial layer; en, endothelium; er, rough endoplasmic reticulum; f, myofilaments; l, lipid drops; m, mitochondria; r, reticulocytes; sc, sarcomeric organisation; se, subepicardial layer; tl, trabecular layer; v, ventricular chamber; z, z band.

epithelial cells on both sides of the neural tube at the neurulation stage (Le Douarin, 1982; Hall and Hörstadius, 1988). There is strong evidence that the cranial NC cells are actively migrating toward the developing pharyngeal arches and the frontonasal process (Morris-Kay and Tan, 1987; Hall and Hörstadius, 1988; Selleck and Bronner-Fraser, 1996). Here, they differentiate into mesenchymal derivatives such as bone, cartilage and muscles (Le Douarin, 1982; Hall and Hörstadius, 1988). Several factors have been implicated in stimulation and guidance of NC migration including cell adhesion molecules (tenascin, fibronectin, laminin) and their receptors (integrins) (Bronner-Fraser, 1993). Humoral factors such as growth factors (Le Douarin et al., 1993; Selleck and Bronner-Fraser, 1996) or neurotransmitters are also implicated in NC migration. Evidence that 5-HT and some of its receptors participate in these processes have also been reported (Moiseiwitsch and Lauder, 1995).

In our experiments of embryos treatment with 5-HT_{2B} antagonist, widespread cell death was detected in the hindbrain, in cranial NC and in disorganised somites (Figs 7, 8). The site of expression of the 5-HT_{2B} receptor in cranial NC cells and the phenotype observed after antagonist treatment, where NC cells present induced-cell death with typical apoptotic nuclei (Fig. 7D,F), suggest a role for 5-HT_{2B} receptors in NC migration, cellular proliferation and/or survival. One possibility is the involvement of 5-HT in the formation of cranial NC cells by epitheliomesenchymal trans-

formation which has been shown to be dependent on PKC activity (Newgreen and Minichiello, 1995), and is under the control of members of the transforming growth factor β superfamily (Selleck and Bronner-Fraser, 1996). Conversely, 5-HT is involved in cranial NC cell migration (Moiseiwitsch and Lauder, 1995), and previous experiments have suggested the involvement of a 5-HT₂-like receptor since the migration of mesenchymal cells can be blocked by methysergide (Lauder and Zimmerman, 1988). The contribution of 5-HT_{2C} receptors in these effects can nevertheless be ruled out, since 5-HT_{2C} knock-out mice do not show any developmental deficits (Tecott et al., 1995). Interestingly, we have detected SERT mRNA (Fig. 1) and paroxetine-binding (Table 1) in extracts from embryos at 8–10 d.p.c., and SERT uptake activity has been localised in the neuroepithelium at the 12 somite-pair stage in rhombomeres r3 and r5 (Shuey et al., 1993). These data along with the observation that apoptotic NC cells are present in the same rhombomeres of chicken embryos at the same stage (Graham et al., 1993; Lumsden and Graham, 1996) indicate that rhombomeres r3 and r5 are associated with apoptotic NC cells and that local uptake activity reduces the levels of 5-HT. It is therefore, tempting to speculate that treatment by 5-HT_{2B}-antagonist mimics the absence of 5-HT stimulation resulting normally from the local uptake of 5-HT and therefore broadens the apoptosis of NC cells (Fig. 8). This would predict that 5-HT acting on the 5-HT_{2B} receptors exerts a trophic action on

cranial NC cells to prevent the induction of programmed cell death.

In conclusion, our data indicate that 5-HT_{2B} receptors participate in the regulation of cranial NC cells, by affecting their migration, cellular proliferation and/or survival, as well as migration and/or inhibition of cellular differentiation of the heart myocardium during early mouse embryogenesis. Investigations with gene targeted (knockout) mice should further validate these findings about the functions of 5-HT_{2B} receptors during early embryonic development.

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ACCELERATED COMMUNICATION

Possible Role of Valvular Serotonin 5-HT_{2B} Receptors in the Cardiopathy Associated with Fenfluramine

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ABSTRACT

Dexfenfluramine was approved in the United States for long-term use as an appetite suppressant until it was reported to be associated with valvular heart disease. The valvular changes (myofibroblast proliferation) are histopathologically indistinguishable from those observed in carcinoid disease or after long-term exposure to 5-hydroxytryptamine (5-HT)₂-preferring ergot drugs (ergotamine, methysergide). 5-HT₂ receptor stimulation is known to cause fibroblast mitogenesis, which could contribute to this lesion. To elucidate the mechanism of "fen-phen"-associated valvular lesions, we examined the interaction of fenfluramine and its metabolite norfenfluramine with 5-HT₂ receptor subtypes and examined the expression of these receptors in human and porcine heart valves. Fenfluramine binds weakly to 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. In contrast,

norfenfluramine exhibited high affinity for 5-HT_{2B} and 5-HT_{2C} receptors and more moderate affinity for 5-HT_{2A} receptors. In cells expressing recombinant 5-HT_{2B} receptors, norfenfluramine potently stimulated the hydrolysis of inositol phosphates, increased intracellular Ca²⁺, and activated the mitogen-activated protein kinase cascade, the latter of which has been linked to mitogenic actions of the 5-HT_{2B} receptor. The level of 5-HT_{2B} and 5-HT_{2A} receptor transcripts in heart valves was at least 300-fold higher than the levels of 5-HT_{2C} receptor transcript, which were barely detectable. We propose that preferential stimulation of valvular 5-HT_{2B} receptors by norfenfluramine, ergot drugs, or 5-HT released from carcinoid tumors (with or without accompanying 5-HT_{2A} receptor activation) may contribute to valvular fibroplasia in humans.

Appetite suppressant medications have been used worldwide for decades for the treatment of obesity. Interest in pharmacological approaches to obesity has been largely driven by an increased cultural pressure for dieting, an ever-increasing fraction of individuals defined as obese, the identification of a causal relationship between obesity and cardiovascular disease and diabetes, and the recognition that nonpharmacological treatments alone have limited efficacy. One of the most widely prescribed anorectic agents was fenfluramine either alone or in combination with the noradrenergic drug phentermine ("fen-phen"). These agents were combined clinically with the presumption that the resulting reduction in the daily dosing of either drug alone would

mitigate untoward side effects while maintaining clinical efficacy. In 1996, the United States approved the use of the *d*-isomer of fenfluramine, dexfenfluramine, for chronic use in the long-term management of obesity. However, this decision was largely rescinded in 1997 when the Food and Drug Administration issued a public health advisory indicating that 33 women who had taken fenfluramine and phentermine in combination had unusual heart valve morphology and regurgitation (Connolly et al., 1997). These observations were histopathologically identical with the fibroplasia seen in carcinoid (with accompanying 5-HT-secreting tumors) or ergotamine-induced valve disease (Connolly et al., 1997; Kulke and Mayer, 1999). Later studies showed that although

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; HEK 293E cells, human embryonic kidney 293 Epstein-Barr nuclear antigen cells; DMEM, Dulbecco's modified Eagle's medium; IA, intrinsic activity; PCR, polymerase chain reaction; PI, phosphoinositide; 5-CT, 5-carboxymidotryptamine; MAP, mitogen-activating protein; FLIPR, fluorescence image plate reader.

the incidence of valve defects may not be as great as initially thought, it appears to be a threat with extended use (>3 mon) of fenfluramine alone or in combination with phentermine (Wee et al., 1998).

In contrast to its well documented 5-hydroxytryptamine (5-HT)-releasing properties, other pharmacological properties of fenfluramine and its metabolite norfenfluramine, particularly regarding their interactions with postsynaptic human 5-HT receptors, are still poorly defined (Curzon et al. 1997). Furthermore, except for a presumptive involvement of 5-HT, no specific mechanisms have been proposed for fenfluramine- and ergot-related cardiopathy. Because the 5-HT₂ receptor subfamily plays a prominent role in the feeding, cardiovascular, and mitogenic effects of 5-HT, we examined the interaction of fenfluramine, norfenfluramine, and ergot drugs with 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. We also measured the mRNA expression levels of these receptor subtypes in porcine and human heart valves.

Experimental Procedures

Materials. [¹²⁵I](1-(4-iodo-2,5-Dimethoxyphenyl)-2-aminopropane (2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA). *myo*-[2-³H]Inositol (15–20 Ci/mmol), [³H]5-HT (122 Ci/mmol), and [³H]carboxymidotryptamine (5-CT; 57 Ci/mmol) were purchased from Pharmacia Amersham (Arlington Heights, IL). Electrophoresis reagents were purchased from Novex (San Diego, CA). Chirally pure isomers, *d* and *l*, of norfenfluramine were synthesized by the Department of Chemical and Physical Sciences at the DuPont Pharmaceuticals Co. Most cell culture supplies were purchased from Life Technologies (Grand Island, NY). All other reagents were purchased from Research Biochemicals Inc. (Natick, MA) or Sigma Chemical Co. (St. Louis, Mo) unless otherwise noted. Stable cell lines were generated by transfecting human embryonic kidney 293 Epstein-Barr nuclear antigen cells (HEK 293E) cells with episomal plasmids containing human 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, or 5-HT_{2C} (VNV edited isoform) cDNA using calcium phosphate as described previously (Fitzgerald et al., 1999).

Radioligand Binding Studies. Radioligand binding assays were conducted in disposable polypropylene 96-well plates (Costar Corp., Cambridge, MA) as described previously (Fitzgerald et al., 1999). The 5-HT_{2A}, 5-HT_{2C}, 5-HT_{1E}, and 5-HT_{1F} assays were initiated by the addition of membrane homogenate in tissue buffer to assay buffer (50 mM Tris · HCl, 0.5 mM EDTA, 10 mM pargyline, 10 mM MgSO₄, 0.05% ascorbic acid, pH 7.5) containing [¹²⁵I](1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane (0.3–0.5 nM final, for the 5-HT_{2A} and 5-HT_{2C} assays) or [³H]5-HT (2–10 nM final, for the 5-HT_{1E} and 5-HT_{1F} assays) with or without competing ligand. The 5-HT_{2B} and 5-HT_{1D} assays were conducted similarly except for the substitution of 4 mM CaCl₂ for the MgSO₄ and the use of [³H]*d*-lysergic acid (2–2.5 nM) or [³H]5-CT (1–2 nM) as the radioligands, respectively. Apparent dissociation constants (*K_i* values) from the competition experiments were calculated using an iterative nonlinear regression curve-fitting program (Prism; GraphPAD Software, San Diego, CA).

Phosphoinositide (PI) Hydrolysis studies. The ability of ligands to stimulate PI hydrolysis was monitored in whole cells expressing recombinant 5-HT receptors as described previously (Fitzgerald et al., 1999). Cells were treated with 0.5 μCi/well *myo*-[³H]inositol for 16 to 18 h, washed with serum/inositol-free Dulbecco's modified Eagle's medium (DMEM) containing 10 mM LiCl and 10 μM pargyline, and incubated for 30 min with the same medium but now containing test compound. Reactions were terminated, and [³H]phosphoinositides were extracted and then separated by anion exchange chromatography as described previously (Fitzgerald et al.,

1999). EC₅₀ values were determined by nonlinear regression analysis with Prism (GraphPAD). *E*_{max} (maximal response) was derived from the fitted curve maxima for each compound. Intrinsic activity (IA) was determined by expressing the *E*_{max} of a compound as a percentage of the *E*_{max} of 5-HT (IA = 1.0).

Measurement of Ca²⁺-Evoked Fluorescence by Fluorescence Imaging Plate Reader (FLIPR). The ability of norfenfluramine to increase intracellular Ca²⁺ was assessed by FLIPR. Cells expressing recombinant 5-HT receptors were seeded onto poly(D-lysine)-coated, 96-well plates (Costar Corp., Cambridge, MA) and incubated overnight or until near confluence was established. Growth medium was then replaced with dye loading buffer (Hanks' solution) containing 20 mM HEPES, 4 μM Fluo-3 (Molecular Probes, Eugene, OR), and 0.04% (w/v) pluronic acid (Molecular Probes) for 60 min at 37°C. Dye buffer was removed and replaced four times with a dye- and pluronic acid-free Hanks solution maintained at 37°C. Cell and drug plates were then placed in a 96-well FLIPR (Molecular Devices, Sunnyvale, CA) and equilibrated for 10 to 15 min at 37°C. Fluorescence readings were taken for 10 s before and 4 min after the agonist addition. Each drug plate contained 6 wells of 3 μM 5-HT that served as an internal standard for the determination of IA.

Measurement of Agonist-Activated Mitogen-Activating Protein (MAP) Kinase Signaling. The ability of ligands to acutely activate (phosphorylate) MAP (Erk1/Erk2) kinase was examined by Western blotting in cells expressing human recombinant 5-HT receptors. Cells were grown to 75% confluency in DMEM containing 10% dialyzed FCS in 100-mm dishes. Cells were serum-starved overnight, and the medium was aspirated and then incubated for 5 min with serum-free DMEM containing test compound. Cells were washed with PBS containing 1 mM orthovanadate, lysed (with 125 mM Tris, 2% SDS, 5% glycerol, 1 mM orthovanadate, and 10 μg/ml aprotinin, leupeptin, and pepstatin), and then sonicated. Lysates (2 μg protein) were boiled, loaded onto 4 to 12% acrylamide gels, electrophoresed, and transferred onto nitrocellulose membranes according to the manufacturer's instructions (Novex, San Diego, CA). Blots were blocked in PBS-Tween (0.05% v/v) containing 2% nonfat milk for 45 min and then incubated overnight (4°C) with antidi-phosphorylated MAP kinase antibody (diluted 1:10,000; Sigma Chemical Co.). After extensive washing (2 h), blots were incubated with secondary antibody (Vector Laboratories, Burlingame, CA; 90 min), washed again in PBS-Tween (2 h), and detected by enhanced chemiluminescence (New England Nuclear).

Expression of 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} mRNA in Porcine and Human Heart Valves. Real-time polymerase chain reaction (PCR) was performed essentially as described (Gibson et al., 1996). Sequences for pig 5-HT_{2A} (Ullmer et al., 1995), 5-HT_{2B} (Ullmer et al., 1995), and GAPDH (accession number AF017079) were obtained from GenBank. Sequences for the 5-HT_{2C} receptor were obtained by reverse transcription-PCR using rat primers, with the resulting products being cloned and sequenced. Primers and probes were designed for 5-HT_{2A} (primers: TGCCCTTCTTCATCACCA and CCGATGACATCCTCGTTGC, probe: TCATGGCCGTCATCTGCAAGAGTCC), 5-HT_{2B} (primers: GCCATTTTCAGTGGATCGTTACATA and AATGCTGTAGCTCGTGAGTTATATTGA, probe: CCATCAAAAGCCAATCCAGGCCA), 5-HT_{2C} (primers: GCACTTCAGGAAATCCAGTG and ATGCTCCTGCGCGGC, probe: TCCCGCGCGTTCCTCGGTC), and GAPDH (primers: GCAATGCTCCTGTACCACC and TGCCGAAGTTGTTCATGGATG, probe: ACTGCTTGGCACCCTGGCC). All probes were labeled at the 5' end with the reporter dye 6-FAM and on the 3' end with the quencher dye TAMRA (Perkin-Elmer/Applied Biosystems, Foster City, CA).

Total RNA was isolated from pig or human aortic valves and mitral valves as described (Davis et al., 1986). Then, 1 μg of total RNA was treated with RNase-free DNase I (Life Technologies, Gaithersburg, MD) and reverse transcribed essentially as described (Huang et al., 1996) with primers and Moloney murine leukemia virus (Clontech, Palo Alto, CA) being added after the DNase I treat-

ment. Each PCR used the cDNA from 100 ng of starting RNA. Valves were obtained from six pigs. The individual leaflets of the aortic valves were combined and pooled for the first three pigs, with data from the entire valve being analyzed. For the three remaining pigs, the individual leaflets of the aortic valves were combined from the three pigs, with the data from the left, right, and middle valve pools being compared. Copy numbers were calculated by comparing the threshold values for each reaction with a standard curve produced using linearized cDNA for the respective genes essentially as described previously (Gibson et al., 1996). The Molecular Probes PicoGreen assay was used to measure the concentration of the linearized DNAs before use in the standard curves as described by the manufacturer. Copy numbers obtained from "no reverse transcriptase" controls were subtracted from the copy numbers obtained for the experimental samples. Expression levels were normalized with GAPDH. All expression levels are relative, because the initial efficiency of the reverse transcription reaction was not accounted for in the copy number calculations.

Results

Radioligand binding experiments were conducted to evaluate the affinities of fenfluramine and norfenfluramine for human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors expressed in HEK 293E cells. The *d*- and *l*-isomers of fenfluramine demonstrated weak affinities ($K_i > 0.7$ – 1.5 μ M) for all three 5-HT receptor subtypes (see Table 1). In contrast, the isomers of norfenfluramine were most potent at the 5-HT_{2B} (27 and 65 nM, *d*- and *l*-, respectively) and 5-HT_{2C} (56 and 99 nM) receptors and less potent at 5-HT_{2A} (187 and 267 nM) receptors. Phentermine was found to be inactive at these receptors at concentrations up to 10 μ M.

In vitro functional assays were then used to assess ligand efficacy and potency. We examined the ability of norfenfluramine to hydrolyze inositol phosphates (IP) and mobilize intracellular Ca²⁺ in whole cells. Both isomers of norfenfluramine were potent high efficacy partial-to-full agonists with respect to their ability to stimulate IP production and increase intracellular Ca²⁺-evoked fluorescence (Table 2 and Fig. 1). As observed in the binding studies, norfenfluramine was most potent at the 5-HT_{2B} receptor, moderately potent at the 5-HT_{2C} receptor, and least potent at the 5-HT_{2A} receptor. Furthermore, although the *d*-isomer of norfenfluramine was consistently more potent than the *l*-isomer, their kinetics of receptor activation (see Fig. 1B) were identical. The isomers of the parent compound, fenfluramine, also behaved as agonists on these measures, but their weak activity precluded an accurate assessment of potency and efficacy (data not shown).

Because the lesion reported in human valves is fibroplasia,

we assessed whether norfenfluramine would activate the MAP kinase (Erk1/2) pathway, a proliferative/mitogenic pathway linked to tyrosine kinase and G protein-coupled receptor activation, including the 5-HT_{2B} receptor (Launay et al., 1996; Lopez-Illasaca, 1998). Cells were stimulated with 1 μ M 5-HT or *d*-norfenfluramine, lysed, and tested for phospho-MAP kinase by Western blotting. This stimulation time (10 min) was shown in a preliminary experiment to elicit maximal levels of activated enzyme (data not shown). Like 5-HT, *d*-norfenfluramine increased the levels of immunoreactive phospho-MAP kinase via all three 5-HT₂ receptor subtypes (Fig. 2).

Last, because the long-term use of ergotamine and methysergide in humans has been associated with a risk for valvular effects similar to those of fenfluramine, we examined their binding affinities and functional properties at human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. Ergotamine (10.9, 28.4, and 19 nM), methysergide (6.8, 0.7, and 1.2 nM), and the principal desmethyl metabolite of methysergide, methylergonovine (1.0, 1.2, and 9.0 nM), possess significant affinity (K_i) for all three 5-HT₂ subtypes (mean K_i values at 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, respectively; $n = 2$). IP hydrolysis assays revealed that methysergide is a silent antagonist and that ergotamine is a partial agonist (IA = 70–90% of 5-HT) at 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. Methylergonovine was found to be a partial agonist at the 5-HT_{2B} and 5-HT_{2A} receptors (40 and 20% of 5-HT, respectively) but an antagonist at 5-HT_{2C} receptors. Because ergots are also known to exert potent vascular effects via 5-HT_{1D}-like receptors, we examined the interactions of methysergide, ergotamine, and methylergonovine at human 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} receptors. Although we confirmed that ergotamine, methylergonovine, and methysergide can show high-to-moderate affinity for these receptors, *d*-norfenfluramine is comparatively weak ($K_i = 5$ – 10 μ M).

We next used real-time PCR to quantify transcript levels of the 5-HT₂ subfamily of receptors in porcine and human aortic and mitral valves. The relative expression levels of the genes were compared within tissues by determining the number of copies present in the starting cDNA relative to the standard curve. We observed 5-HT_{2A} and 5-HT_{2B} receptor mRNA levels that were significantly higher than 5-HT_{2C} receptor levels in oligo(dT) primed cDNA from both aortic and mitral valves (Fig. 3). After normalizing between experiments with GAPDH levels, we observed between 13 and 20 copies of 5-HT_{2C} receptor transcript in 100 ng of total RNA from aortic and mitral valves relative to plasmid-based standard curves. In aortic valves, 5-HT_{2A} and 5-HT_{2B} receptor transcripts

TABLE 1

Norfenfluramine, but not the parent molecule fenfluramine, potently binds to the human 5-HT_{2B} receptor and other members of the 5-HT₂ receptor subfamily

Competition experiments were conducted as described in *Materials and Methods*. Depicted are mean \pm S.E. K_i values of enantiomers of fenfluramine and norfenfluramine versus [¹²⁵I]DOI for the 5-HT_{2A} and 5-HT_{2C} receptor or [³H]LSD binding for the 5-HT_{2B} receptor (from three to six independent experiments).

Receptor Subtype	K_i			
	<i>d</i> -Fenfluramine	<i>l</i> -Fenfluramine	<i>d</i> -Norfenfluramine	<i>l</i> -Norfenfluramine
	nM			
5-HT _{2A}	2470 \pm 240	1430 \pm 330	187 \pm 10	267 \pm 16
5-HT _{2C}	2080 \pm 480	1620 \pm 340	56 \pm 19	99 \pm 12
5-HT _{2B}	3920 \pm 830	680 \pm 16	27 \pm 7	65 \pm 23

were expressed at 757- and 375-fold higher levels than 5-HT_{2C} levels, respectively. Similarly in mitral valves, 5-HT_{2A} and 5-HT_{2B} were expressed at 440- and 360-fold higher levels than 5-HT_{2C} levels. We also compared data for the left, right, and middle leaflets of the aortic valves and saw no differences in 5-HT receptor expression among the different leaflets. We repeated the real-time PCRs using random-primed and gene-specific primed cDNA to assure ourselves that oligo(dT) priming did not bias our results. The relative expression patterns were consistent between datasets. When compared with 5-HT_{2C} receptor expression, 5-HT_{2A} and 5-HT_{2B} receptors were shown to be expressed at approximately 750 and 250 times higher levels in random primed aortic valve cDNAs. In mitral valve cDNA, 5-HT_{2A} and 5-HT_{2B} were expressed at levels that were 250- and 150-fold higher than 5-HT_{2C} levels. In a limited number of experiments using gene-specific primers for the reverse transcription, we obtained similar results. However, the limiting amount of RNA that we had available made the use of independent reverse transcription reactions for each amplification impractical. The 5-HT_{2C} primer/probe reagents efficiently detected more than 10⁵ copies of 5-HT_{2C} in choroid plexus cDNA, with the assay being linear between 10 copies and 10⁷ copies per PCR. Therefore, inefficiencies in the assay were not responsible for the low 5-HT_{2C} levels observed in the valve RNAs.

We repeated the real-time PCR experiments with RNA isolated from human valves that had been obtained from patients undergoing valve replacement. The human valves had high levels of calcification and yielded low amounts of poor quality RNA. However, the data obtained from human valves were consistent with that of the pig. We observed 200 to 700 copies of 5-HT_{2A} and 5-HT_{2B} transcripts in human cDNA samples, respectively, whereas 5-HT_{2C} levels were below the level of detection for the assay (data not shown).

TABLE 2

The enantiomers of norfenfluramine are potent agonists at the recombinant human 5-HT_{2B} receptor

Potencies (EC₅₀ values) and efficacies [intrinsic activity (IA)] of fenfluramine and norfenfluramine at eliciting inositol phosphate (IP) hydrolysis and intracellular Ca²⁺ mobilization. Intrinsic activities were calculated by comparing net maximal stimulation of [³H]IP hydrolysis or Ca²⁺-mediated fluorescence by norfenfluramine with the net maximal effects elicited by the full agonist 5-HT at the human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. Mean potencies for 5-HT in stimulating IP production via human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors were 178.3, 39.4, and 21.7 nM, respectively. Mean potencies for 5-HT in stimulating Ca²⁺ mobilization via human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors were 30.9, 2.8, and 21.2 nM, respectively. Mean potencies and IAs (±S.E.) were derived from three to five independent experiments.

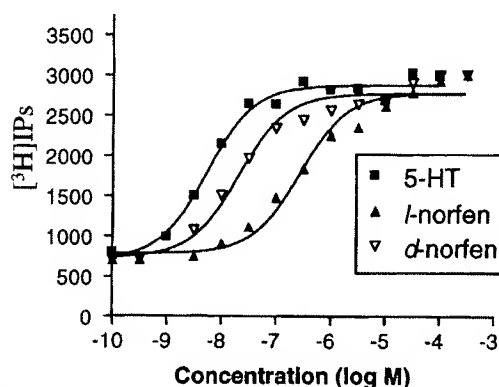
Functional Assay	EC ₅₀ (IA)	
	<i>d</i> -Norfenfluramine	<i>l</i> -Norfenfluramine
	nM	
5-HT _{2A} receptor		
IP hydrolysis	3,100 ± 330 (0.7 ± 0.02)	26,600 ± 4,290 (0.5 ± 0.01)
Ca ²⁺ mobilization	720 ± 77 (0.8 ± 0.04)	1,990 ± 320 (0.4 ± 0.08)
5-HT _{2C} receptor		
IP hydrolysis	190 ± 16 (0.9 ± 0.03)	727 ± 45 (0.8 ± 0.05)
Ca ²⁺ mobilization	300 ± 29 (0.9 ± 0.09)	995 ± 56 (0.8 ± 0.05)
5-HT _{2B} receptor		
IP hydrolysis	24 ± 3.7 (0.9 ± 0.01)	292 ± 50 (0.8 ± 0.02)
Ca ²⁺ mobilization	23 ± 4 (1.0 ± 0.1)	239 ± 39 (0.7 ± 0.04)

Discussion

The goal of the present study was to examine the pharmacology of fenfluramine and its metabolite, norfenfluramine, with 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, a subfamily of

A.

IP hydrolysis via the 5-HT_{2B} receptor



B.

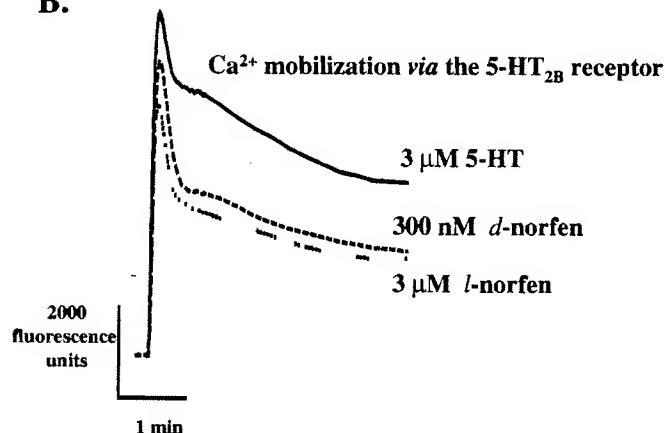


Fig. 1. A, norfenfluramine-mediated stimulation of PI hydrolysis in adherent HEK 293E cells expressing the human 5-HT_{2B} receptor. Cells were incubated with different concentrations of ligand for 30 min. The reactions were terminated, and inositol phosphates were isolated using anion exchange chromatography. B, comparison of the time course for calcium-induced fluorescence evoked by 5-HT, *d*-norfenfluramine, and *l*-norfenfluramine in HEK 293E cells expressing the 5-HT_{2B} receptor. Refer to Table 2 for a detailed presentation of the functional data. Each depicted curve or trace is representative of three experiments with similar results.

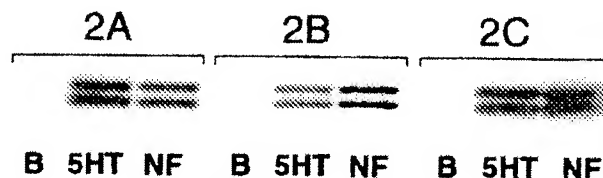


Fig. 2. Activation of the MAP kinase by 1 mM 5HT or norfenfluramine in cells expressing 5-HT_{2A}, 5-HT_{2B}, or 5-HT_{2C} receptors as assessed by Western blotting. Cells were treated with 5-HT or *d*-norfenfluramine (NF) for 10 min, lysed, and loaded onto SDS-polyacrylamide gels. Nitrocellulose filters were blotted for immunoreactive MAP kinase using a monoclonal antibody that recognizes the diphosphorylated (activated) form of MAP kinase.

5-HT receptors implicated in the feeding, cardiovascular, and mitogenic effects of 5-HT.

Radioligand binding experiments were first conducted to determine the affinities of fenfluramine and norfenfluramine for human recombinant 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. The *d*- and *l*-stereoisomers of fenfluramine demonstrated weak affinities ($K_i > 0.7$ – $1.5 \mu\text{M}$) for all three 5-HT receptor subtypes. In contrast, the isomers of norfenfluramine exhibited high-to-moderate affinity for 5-HT_{2B} (27 and 65 nM, *d*- and *l*-isomer, respectively) and 5-HT_{2C} receptors (56 and 99 nM) but lesser affinity for 5-HT_{2A} receptors (187 and 267 nM). Based on reported steady-state blood concentrations of norfenfluramine after average clinical doses in humans (Caccia et al., 1985), norfenfluramine could sufficiently occupy 5-HT_{2C} and 5-HT_{2B} receptors and contribute to the pharmacological and toxicological actions of fenfluramine. Phentermine, the other component of the "fen-phen" combination, was found to be inactive at these receptors.

In vitro functional assays were used to assess ligand efficacy and potency. We examined the ability of norfenfluramine to stimulate hydrolysis of IP and the subsequent increase in intracellular Ca^{2+} in whole cells. Stereoisomers of norfenfluramine were potent agonists with respect to their ability to stimulate IP production and increase intracellular Ca^{2+} . Norfenfluramine was most potent at the 5-HT_{2B} receptor, was moderately potent at the 5-HT_{2C} receptor, but was very weak in activating the 5-HT_{2A} receptor. We intentionally used lines expressing modest levels of receptor and exhibiting low receptor reserve. The intrinsic activities of other known 5-HT agonists (e.g., partial agonists, *m*-chlorophenylpiperazine and *d*-lysergic acid) at these receptors were shown previously to compare favorable with efficacies observed in native tissues (Fitzgerald et al., 1999).

Because the lesion underlying the cardiac valvular regurgitation in humans is fibroplasia (myofibroblast proliferation), we assessed whether norfenfluramine would activate the MAP kinase (ERK1/2) pathway, a mitogenic pathway linked to tyrosine kinase and G protein-coupled receptor activation (Lopez-Illasaca, 1998). Like 5-HT, *d*-norfenfluramine increased the levels of immunoreactive phospho-MAP kinase via all three 5-HT₂ receptor subtypes. The prolifera-

tive potential of the 5-HT₂ subfamily of 5-HT receptors was demonstrated when it was shown that the 5-HT_{2C} receptor could transform NIH3T3 fibroblasts (Julius et al., 1989). More recently, activation of the *ras*-MAP kinase pathway has been implicated in the mitogenic and transforming properties of the 5-HT_{2B} receptor expressed at moderate densities in fibroblasts and endogenously in carcinoid tumors (Launay et al., 1996). The 5-HT_{2B} receptor also mediates important trophic functions in cardiovascular morphogenesis. Embryonic expression of 5-HT_{2B} predates that of the 5-HT_{2A} and 5-HT_{2C} receptors and is localized to the heart primordia and neural fold before neural tube closure (Choi et al., 1998).

Because fenfluramine-induced valvular changes are histopathologically identical with those seen after chronic exposure to 5-HT₂ receptor-preferring ergots (ergotamine, methysergide), we examined their affinities, along with that of the active metabolite of methysergide, methylergonovine, at the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. Methylergonovine concentrations in plasma have been reported to be substantially higher than the parent drug after the oral administration of methysergide; it is postulated to be the main contributor of methysergide efficacy and side effects (Muller-Schweinitzer and Tapparelli, 1986). All three ergot compounds exhibited high affinity for all three 5-HT₂ receptor subtypes (see also Kursar et al., 1992; Newton et al., 1996; Schmuck et al., 1996). IP hydrolysis assays revealed that methysergide is a silent antagonist and ergotamine is a partial agonist at 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. Methylergonovine was found to be a partial agonist at the 5-HT_{2B} and 5-HT_{2A} receptors but an antagonist at 5-HT_{2C} receptors.

Although these data collectively are consistent with the hypothesis that the valvular lesions are due to stimulation of myofibroblast mitogenesis via activation of 5-HT_{2B} receptors, the interaction of norfenfluramine and ergots with other receptor subtypes may also contribute to these changes. Because ergots are known to exert potent vascular effects via 5-HT_{1D}-like receptors, we examined the interactions of methysergide, ergotamine, methylergonovine, and norfenfluramine at human 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} receptors. We confirmed that although ergotamine, methylergonovine, and methysergide can exhibit high-to-moderate affinities for

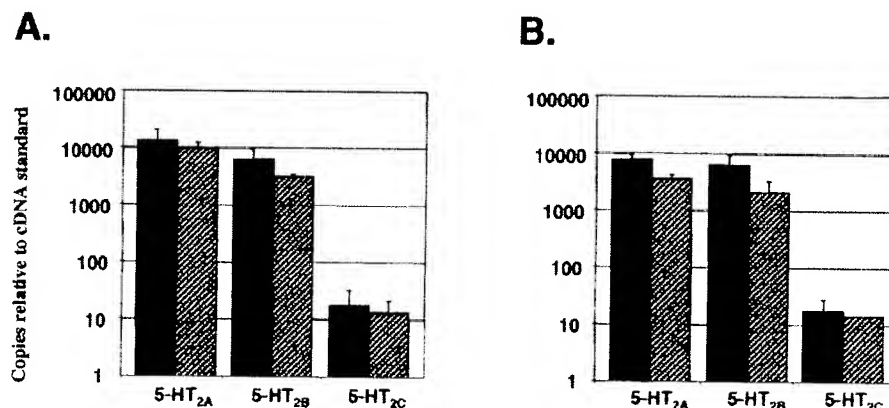


Fig. 3. Real-time PCR was performed as described in the test using oligo(dT) or random primed cDNA. Data are shown as mean relative copies (\pm S.E.) per 100 ng of starting RNA for aortic (A) and mitral (B) valves. Solid columns represent data from random primed cDNA experiments, and hatched columns denote oligo(dT) data. Expressions for 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors are shown as relative copy numbers because they were calculated directly from standard curves generated with cDNA clones.

these receptors, *d*-norfenfluramine is comparatively weak ($K_i = 5\text{--}10\ \mu\text{M}$). This inactivity of norfenfluramine along with the observation that tryptamines used clinically for migraine (e.g., sumatriptan) interact with 5-HT_{1D}-like receptors (Johnson et al., 1998) but do not cause valve defects suggests that fenfluramine- and ergot-related valve defects are not mediated by a common 5-HT_{1D} mechanism. Furthermore, sumatriptan is also inactive ($>10\ \mu\text{M}$) at 5-HT_{2B} receptors (Schmuck et al., 1996). Some ergot drugs (e.g., bromocriptine) can activate dopamine receptors, and are used chronically to treat Parkinson's disease and hyperprolactinemia. However, bromocriptine and dopamine receptor agonists in general have not been associated with valvular heart disease.

We next used real-time PCR to quantify mRNA levels of the 5-HT₂ subfamily of receptors in porcine aortic and mitral valves. 5-HT_{2A} and 5-HT_{2B} receptor mRNA levels were more than two orders of magnitude higher than 5-HT_{2C} receptor transcript levels in oligo(dT)-primed cDNA from both aortic and mitral valves. We repeated the real-time PCR amplifications using random-primed and gene-specific primed cDNA to ensure that oligo(dT) priming did not bias our results. The overall patterns of expression were consistent among the various methods: 5-HT_{2B} and 5-HT_{2A} transcripts were abundant relative to 5-HT_{2C} transcript levels, which were extremely low to undetectable. Determination of absolute copy numbers for each gene is difficult because we have no measure of the reverse transcription efficiency. However, even if we assume that it is as low as 1%, the expression levels of the 5-HT_{2C} receptor would be predicted to be less than one transcript per cell in both mitral and aortic valves. Real-time PCR experiments with RNA isolated from human valves that had been obtained from patients undergoing valve replacement yielded similar results. It is unclear what cell types reflect the 5-HT₂ receptor transcript expression pattern in the heart valves because RNA from whole tissues was used; future work using *in situ* hybridization or immunohistochemical techniques may help resolve this question.

The combined data suggest that the agonist interaction of norfenfluramine with 5-HT_{2B} receptors may contribute to the proliferative valvular heart valve disease seen with fenfluramine. For both fenfluramine- and methysergide-induced cardiomyopathy, our analysis suggests that the primary metabolite of these drugs may be responsible for the myofibroblast proliferation characteristic of this toxicity. Although all three 5-HT₂ receptor subtypes could trigger the cell proliferation characteristic of ergot and norfenfluramine cardiopathy, the 5-HT_{2B} receptor presents with the most compelling dataset for this role. The relative absence of 5-HT_{2C} receptor transcripts in heart valves and the complete lack of agonist activity by methysergide or its metabolite methylergonovine at the 5-HT_{2C} receptor argue against a role for this receptor subtype in the common valvular pathology caused by ergots and fenfluramine. In contrast, the 5-HT_{2A} receptor transcript is abundantly expressed in heart valves, and both ergots and norfenfluramine show moderate-to-potent affinity for this receptor subtype. Steady-state blood levels of free (plasma protein unbound) norfenfluramine ($\approx 48\ \text{nM}$, assuming free concentration equates with levels at receptor sites) in humans (Caccia et al., 1985; Spinelli et al., 1988) suggest a far lesser role for 5-HT_{2A} receptors, because it exceeds the midpoint for activation of the 5-HT_{2B} receptor (i.e., $\approx 24\ \text{nM}$)

although remaining well below levels (i.e., $>700\ \text{nM}$) capable of significantly activating the 5-HT_{2A} receptor. However, this argument rests solely on *in vitro* potency differences at recombinant receptors. We cannot exclude a role for the 5-HT_{2A} receptor without a comprehensive pharmacological analysis (e.g., protein expression and reserve) of native receptors in heart valves. Characterization of valvular 5-HT_{2B} and 5-HT_{2A} receptors was judged impractical for the present study because of the number of pigs needed to obtain sufficient numbers of acutely isolated myofibroblasts in culture. Last, we cannot exclude the possibility that interactions with other molecular targets (known or orphan) are contributing factors. Nevertheless, the pharmacological arguments presented herein suggest that direct agonist actions of norfenfluramine at the 5-HT_{2B} receptor may contribute to this valvular lesion.

In summary, agonistic interactions of norfenfluramine with 5-HT_{2B} receptors, perhaps with modest 5-HT_{2A} receptor activation, may directly induce mitogenic activities responsible for the cell proliferation and the cardiotoxic effects of fenfluramine in humans. 5-HT_{2B} receptor activation could also contribute to explain the cardiopathy seen in carcinoid disease as well as after treatment with ergots. Corroborating studies are necessary to validate the mechanistic involvement of this receptor and perhaps identify other genetic and environmental factors that predispose certain individuals for this lesion. This understanding will ultimately lead to the design of safer medicines for future therapeutic use.

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Serotonin 2B receptor is required for heart development

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Several lines of evidence suggest that the serotonin (5-hydroxytryptamine, 5-HT) regulates cardiovascular functions during embryogenesis and adulthood. 5-HT binds to numerous cognate receptors to initiate its biological effects. However, none of the 5-HT receptor disruptions in mice have yet resulted in embryonic defects. Here we show that 5-HT_{2B} receptor is an important regulator of cardiac development. We found that inactivation of 5-HT_{2B} gene leads to embryonic and neonatal death caused by heart defects. 5-HT_{2B} mutant embryos exhibit a lack of trabeculae in the heart and a specific reduction in the expression levels of a tyrosine kinase receptor, ErbB-2, leading to midgestation lethality. These *in vivo* data suggest that the Gq-coupled receptor 5-HT_{2B} uses the signaling pathway of tyrosine kinase receptor ErbB-2 for cardiac differentiation. All surviving newborn mice display a severe ventricular hypoplasia caused by impaired proliferative capacity of myocytes. In adult mutant mice, cardiac histopathological changes including myocyte disarray and ventricular dilation were consistently observed. Our results constitute genetic evidence that 5-HT via 5-HT_{2B} receptor regulates differentiation and proliferation of developing and adult heart. This mutation provides a genetic model for cardiopathy and should facilitate studies of both the pathogenesis and therapy of cardiac disorders in humans.

neuregulin | knockout | proliferation | transactivation

Serotonin (5-hydroxytryptamine) (5-HT) first isolated as a vasoconstrictor from blood was later identified in the central nervous system (CNS). It is found in three main areas of the body: the intestinal wall, platelets, and CNS. The functions of 5-HT in CNS as a neurotransmitter are numerous and appear to involve control of appetite, sleep, memory and learning, temperature regulation, mood, behavior (including sexual and hallucinogenic behavior), and endocrine regulation (1). Peripherally, 5-HT, which is stored in platelets, appears to play a major role in homeostasis, blood pressure regulation, cardiovascular functions (2), motility of the gastrointestinal tract (3), and carcinoid tumor pathology (4). Independently of its location in adults, 5-HT also has been detected during zygotic cleavage divisions, gastrulation and neurulation in embryos of sea urchins, frogs, chicken, and *Drosophila*. The presence of 5-HT and its receptors in early embryogenesis and the ability of 5-HT-specific pharmacological agents (5) to interfere with embryonic development suggested that early embryos use 5-HT before the onset of neurogenesis to regulate cell proliferation and/or morphogenetic movements (6, 7). Furthermore, 5-HT has been suspected for years to regulate craniofacial and cardiovascular morphogenesis: In embryos grown in the presence of either a high concentration of 5-HT or 5-HT-specific reuptake inhibitors, a decreased proliferation of myocardium, cardiac mesenchyme, and endothelium has been reported, indicating that 5-HT may regulate proliferation in the embryonic heart (8).

These biological actions of 5-HT are mediated by numerous cognate receptors. It now appears that there are at least 15 receptor subtypes that belong to four populations: 5-HT_{1/5}, 5-HT₂, 5-HT₃, and 5-HT_{4/6/7} subtypes (9). This diversity has

limited the use of a pharmacological approach toward the understanding of the specificity of these various receptors. An alternative way of investigation is genetic inactivation of genes encoding these receptors. Until now, the phenotype obtained by homologous recombination of 5-HT receptor genes (5-HT_{1A}, 5-HT_{1B}, or 5-HT_{2C}) has led exclusively to behavioral abnormalities without reported morphological defects in CNS (10). The receptors required for the peripheral 5-HT functions still need to be identified.

The 5-HT₂ receptor family comprises three subtypes: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. The 5-HT_{2B} receptor belongs to the G protein-coupled seven transmembrane-spanning receptor family. The 5-HT_{2B} receptor is expressed in embryonic (11) and adult (12) cardiovascular tissues, gut and brain from the rat, mouse, and human species. Binding of 5-HT to this receptor activates Gαq/11 protein, thereby activating phospholipase C, which initiates a rapid release of inositol trisphosphate, resulting in an increase in intracellular calcium levels in transfected cells. Recently, this receptor has been shown to activate the phospholipase A2 (13) and nitric oxide synthases (14) in transfected and endogenously 5-HT_{2B}-expressing cells. Agonist stimulation of 5-HT_{2B} receptor subtype also causes a rapid and transient activation of the protooncogene product p21^{ras} (15) and p42^{mapk}/p44^{mapk} mitogen-activated protein kinases and transduces a mitogenic signal by activating the nonreceptor tyrosine kinase c-Src and the receptor tyrosine kinase platelet-derived growth factor receptor (16) in mouse fibroblast cells. 5-HT_{2B} receptor expression has been shown to induce tumors in nude mice and has been detected at a high level in human carcinoid tumors (15). By using a mouse embryo culture technique, treatment with broad spectrum 5-HT₂ receptor antagonists resulted in severe morphological defects of neuroectodermal and mesodermal derivatives including ventricular myocardium (11). These differentiation processes may be controlled, at least in part, by the mitogenic properties of 5-HT_{2B} receptor and integration of receptor tyrosine kinase-signaling pathways. However, the molecular mechanism by which 5-HT regulates embryonic development is currently unknown because none of the inactivated 5-HT receptors has yet been reported to induce obvious developmental defects other than behavioral defects (10).

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Abbreviations: CNS, central nervous system; dpc, day postcoitum; ES, embryonic stem; 5-HT, serotonin (5-hydroxytryptamine); TEM, transmission electron microscopy.

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Here it is shown that targeted inactivation of the 5-HT_{2B} receptor gene leads to disturbed heart development, thereby resulting in embryonic lethality and death in neonatal mice. Our results identify a 5-HT_{2B} receptor-mediated pathway by which 5-HT interacts with ErbB-2 to control differentiation in developing heart. Together, these results reveal 5-HT via 5-HT_{2B} receptor as an important regulator of cardiac myocyte differentiation and proliferation.

Materials and Methods

5-HT_{2B} Receptor Gene Targeting and Genotype Analysis. The targeting vector was constructed with the selective bacterial *neo* gene introduced into the exon 2 sequence of the 5-HT_{2B} receptor locus. This resulted in a nonfunctional allele of 5-HT_{2B} because it interrupts the protein reading frame. One recombination event was obtained after screening of $\approx 4,000$ embryonic stem (ES) cells clones. Southern blot analysis on the recombined clone with a probe derived from the *neo* sequence revealed that it contained a single expected 16-kbp fragment in a *Hpa*I restriction enzyme digestion excluding multiple insertion of the targeting vector (not shown). From this recombined 5-HT_{2B} ES cells strain, 13 chimeric animals were created by injection into C57/BL6 blastocyst embryos. These chimeras were bred to either the C57/BL6 or 129/PAS mouse strain to obtain germ-line transmission. After transfection of the 5-HT_{2B} targeting vector into 129/PAS ES cells, recombined clones were analyzed by Southern blot with a probe (P) located 3' to the sequence present in the targeting vector. Tail DNAs from newborn mice derived from intercrosses between heterozygous mice were digested with *Bgl*II and analyzed by Southern blot with probe P. All data have been obtained with mice bearing the mutation in a pure 129/PAS background, but similar defects have been observed in a mixed C57/BL6–129/PAS background.

Analysis of Mouse 5-HT₂ Receptor Expression. Membrane proteins prepared from 6-week-old mice heart ventricles ($n = 4$) or stomach ($n = 5$) were analyzed by binding studies with specific tritiated antagonists of the 5-HT_{2B} ([³H]LY266070), 5-HT_{2A} ([³H]MDL100907), or 5-HT_{2C} ([³H]Mesulergine) receptors as described (11).

Morphological Analysis of Mouse Embryos. Electron microscopy and histological experiments were performed as described (11). The hearts were fixed in paraformaldehyde, dehydrated and paraffin-embedded, then sectioned (7 μ m) transversely from the apex, and sections were examined after Masson's trichrome staining. The hearts immersed in a cardioplegic solution (25 mM KCl/5% dextrose in PBS) to ensure complete myocardial relaxation before the hearts were fixed for transmission electron microscopy (TEM). Immunocytochemical labeling of embryos with a 5-HT_{2B}-specific antibody on cryosections or anti-ErbB-2 (SC-284)-specific antibody (Santa Cruz Biotechnology) on paraffin sections was performed as described (11). Whole-mount immunohistochemical staining of embryos for developing blood vessels with antiplatelet endothelial cell adhesion molecule (PharMingen 01951D) antibody were performed as described (11). Western blot analysis for ErbB-2 and platelet-derived growth factor receptor- β (SC-432) was performed in the whole embryo or cell lysate as described (16).

Analysis of ErbB-2 mRNA Expression. Semiquantitative reverse transcription-PCR was performed on 1 μ g of total RNA for 30 cycles at an annealing temperature of 55°C. Normalization was performed by using RNA for elongation factor 1 α . The oligonucleotides used for ErbB-2 are 5'-cgtgctagtcgaagatcccaacc and 5'-tactcttcagcatcgaccagctc; for elongation factor 1 α , 5'-acaaactgaaagctgagcgtg and 5'-gtgcattccacagacttgac; and for

ErbB-4, 5'-tgaacaatgtgatggcaggtgc and 5'-cacgaagttatgagga-catttc.

Analysis of Proliferation Rate. Ventricular cardiomyocytes were prepared from the hearts of newborn and 9.5-day postcoitum (dpc) embryos from 5-HT_{2B} mutant and wild-type mice according to Adams *et al.* (17). The hearts were excised and kept in the buffer containing 113 mM NaCl/4.7 mM KCl/0.6 mM NaH₂PO₄/0.6 mM Na₂HPO₄/1.2 mM MgSO₄/12 mM NaHCO₃/20 mM glucose/10 mM Hepes. The atrium was removed and the ventricle was minced and then cells were dispersed by gentle agitation or gentle pipetting (for embryos). After 15 min of enzymatic digestion with collagenase type II and pancreatin at 37°C, myocytes were pelleted and washed, and differentially plated for 1 h to eliminate remaining nonmyocytes. Cells then were briefly pelleted at low speed (800 g) and rinsed twice in buffer. Rod-shaped cardiac myocytes were plated in culture medium on gelatin (2%)-precoated 8-well chamber slides in the presence of antimitotic (cytosine arabinoside) overnight. Cardiomyocytes were kept in DMEM with 10% FCS and antibiotics at a density of 10³ cells per well. Approximately 95% of the cells displayed spontaneously contractile activity in culture and were verified by myosin heavy chain staining.

Thymidine incorporation was detected in cardiomyocytes as described (16). Briefly, quiescent cells (24-h serum starved) were treated with 5-HT (1 μ M) or neuregulin (heregulin) (25 μ g/ml) for 16 h, and 0.5 mCi of [³H]thymidine was added to the culture during the last 4 h of incubation. The free radioactivity was washed in 5% trichloroacetic acid, and the incorporated radioactive thymidine was quantified by scintillation counting. BrdUrd incorporation was performed by using a BrdUrd-labeling and staining kit (Boehringer Mannheim). Newborn mice were i.p. injected with BrdUrd; 4 h after injection, hearts were dissected and cardiac cryosections were stained with BrdUrd antibody. The sections were washed with water and mounted in 4',6-diamidino-2-phenylindole-containing medium. Slides were kept at 4°C in the dark.

Results and Discussion

To generate 5-HT_{2B} mutant mice, we produced ES cells in which one 5-HT_{2B} allele was disrupted by homologous recombination (Fig. 1a). After injection into the blastocyst of the recombined ES cells, we obtained chimeras that transmitted the mutation. The genotype of 5-HT_{2B} recombinant mice was determined by Southern blot analysis of tail DNA (Fig. 1b). The lack of functional 5-HT_{2B} receptors was confirmed at the RNA level by reverse transcription-PCR amplification (data not shown) and the protein level by binding experiments with receptor subtype-specific-labeled antagonists. In addition to a loss of 5-HT_{2B} receptor expression, no compensatory overexpression of either 5-HT_{2A} or 5-HT_{2C} receptors in heart and stomach tissues from 5-HT_{2B} homozygous mice was detected (Fig. 1c). Heterozygous 5-HT_{2B} mutant intercrosses resulted in a frequency of homozygous pups in newborns of only 16.7%, significantly different from the expected 25% ($P < 0.05$, $n = 120$, according to an unpaired *t* test), suggesting some embryonic lethality (Fig. 1d). When embryos were examined, a Mendelian ratio of homozygous mutant was seen up to 10.5 dpc. The surviving curve for mutants shows the periods of lethality before 11.5 dpc and during the first week of life (Fig. 1e). Nevertheless, the growth of some mutant embryos was clearly retarded at 9.5 and 10.5 dpc, exhibiting enlarged hearts and a pooling of blood in the abdominal area. Closer examination revealed that this was caused by blood leakage into the pericardial cavity (Fig. 2a). Surviving homozygous embryos at 12.5 dpc exhibited developmental delays and paler color than wild-type littermates (Fig. 2b). Because the 5-HT_{2B} receptor is expressed in embryonic (11) and adult (12) cardiovascular tissues, including myocardial, endothelial, and

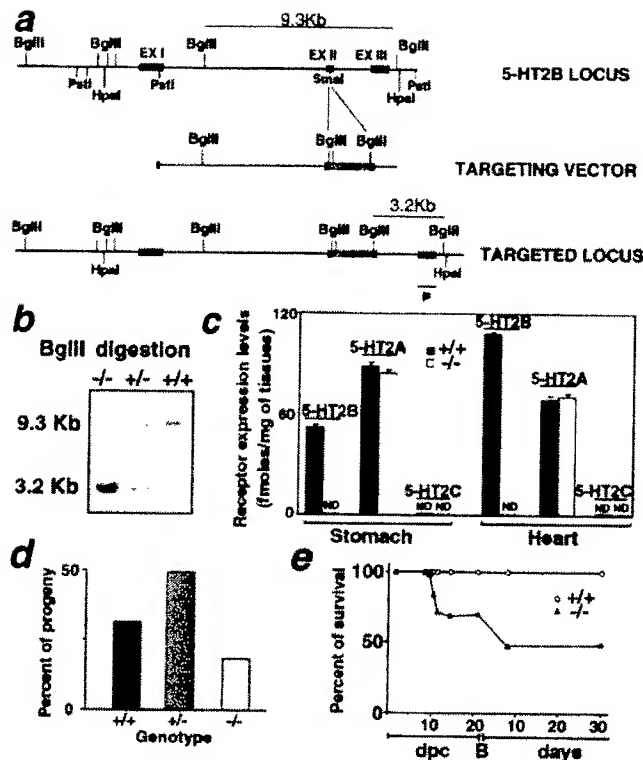


Fig. 1. Targeted disruption of the 5-HT_{2B} receptor gene. (a) (Top) The restriction map of the 5-HT_{2B} receptor genomic locus of interest; (Middle) the targeting construct; and (Bottom) the mutated locus after homologous recombination. (b) The wild-type (+/+), heterozygous (+/-), and homozygous mutant (-/-) alleles were detected by Southern blot analysis. The probe (P) located outside of the construct detects a 9.3-kbp *Bgl*II fragment from wild-type DNA whereas it is reduced to 3.2-kbp in the homologous recombined allele. (c) The analysis of receptor membrane proteins by binding assay revealed a complete loss of 5-HT_{2B}-specific sites in stomach and heart tissues of mutant mice. The maximal amount of binding sites is expressed as fmol specific binding/mg tissues \pm SEM ($n > 4$). ND, no specific binding detected. (d) Genotyped progeny from 5-HT_{2B} +/- intercrosses showed that the homozygous genotype did not exhibit a Mendelian ratio, indicating some embryonic lethality among the homozygous. The percent of progeny is calculated from 120 newborn mice from 14 different litters. The homozygous mutant (-/-) (22 out of 120) represented 66% of the expected viable calculated, assuming sum of +/+ and +/- is 75%. (e) Percentage of homozygous survival before and after the birth in the progeny of heterozygous crosses. Before birth (B), the percent of survival is estimated from more than four litters of heterozygous crosses ($n > 30$), and a Mendelian ratio is observed until 10.5 dpc.

vascular smooth muscle cells, we reasoned that defects observed in the mutant embryos might reflect problems in either vascular organization or cardiac morphogenesis. Whole-mount immunohistochemical staining of 10.5 dpc mutant embryos by using a mAb against platelet endothelial cell adhesion molecule as a marker of developing blood vessels revealed no gross defects in vascular patterning (Fig. 2c). Histological analysis of hearts from 5-HT_{2B} mutant embryos revealed defects in the subepicardial layer, and a lack of trabecular cells in the ventricle (Fig. 2d and e). However, the endocardial cushion appeared to be normal or only slightly reduced in size in some mutant embryos. The atrium and the outflow tract of the mutants appeared normal. The myocardial abnormalities in the 5-HT_{2B} mutants correlate with the pattern of 5-HT_{2B} receptor expression in the compact zone and the myocardial trabeculae (Fig. 3c). TEM studies revealed that all observed mutant hearts ($n = 6$) at 9.5 dpc display premature differentiation of sarcomeres (the contractile unit

found in differentiated myocardial cells) within the compact zone (Fig. 2f), even within regions of the heart showing otherwise nearly normal trabeculation. The most severe reduction in the thickness of the myocardium is likely to induce myocardial rupture, resulting in escape of blood into the pericardium and death. Because we reported that 5-HT_{2B} receptor transduces mitogenic signals in fibroblasts (15, 16), impaired differentiation, migration, and/or proliferation in the heart of 5-HT_{2B}-mutant embryos probably accounts for the midgestation lethality. 5-HT developmental action has been suspected to trigger embryopathies including cardiac pathology observed in embryos from phenylketonuria patients who exhibit a low level of 5-HT in their blood (18).

Identification of factors controlling myocardial differentiation and proliferation is of great importance for understanding the pathogenesis of congenital heart diseases. Remarkably, embryonic heart defects reported in the mice lacking neuregulin or its receptors, ErbB-2 (HER-2/*neu*) and ErbB-4 are similar to those noted in 5-HT_{2B} embryos (19). Expression studies showed that neuregulin is expressed in the endothelial cells of the endocardium and both ErbB-2 and ErbB-4 are localized into the ventricular wall of the myocardium (20). In ErbB-2 or ErbB-4 mutant mice, strong defects in ventricular trabeculation that are nearly identical to that seen in most severely affected 5-HT_{2B} mutant embryos, trigger death at 10.5 dpc. This similarity suggested possible interaction between these two signaling pathways. To this purpose, we analyzed the ErbB-2 and ErbB-4 expression in 5-HT_{2B} mutants. Interestingly, at 9.5 dpc, mutant embryos exhibit a significant reduction in ErbB-2 mRNA, whereas ErbB-4 mRNA expression is not altered. Immunohistochemical analysis revealed that the pattern of ErbB-2 expression in the wild-type heart (Fig. 3a and b Right) is overlapping that of 5-HT_{2B} receptor expression (Fig. 3c) but is significantly reduced in 5-HT_{2B} mutant embryo hearts (Fig. 3a and b Left). Moreover, Western blot analysis revealed a significant reduction in ErbB-2 protein levels in the mutant embryo extracts at 9.5 dpc (Fig. 3d and e). However, another growth factor receptor expressed in heart at this period, such as the platelet-derived growth factor receptor protein remains unchanged (Fig. 3e). Reduction in ErbB-2 protein expression becomes less significant at 11.5 dpc, i.e., after the period of lethality, because the most affected 5-HT_{2B} mutant embryos are resorbed at 11 dpc (Fig. 3e). Because ErbB-2 can be phosphorylated (transactivated) by activation of G protein-coupled receptors (21), this cardiac phenotype could result from a lack of ErbB-2 transactivation by 5-HT_{2B}. However, an ErbB-2 regulation at the transcriptional level seems more likely because both mRNA and protein levels of ErbB-2 are reduced in 5-HT_{2B} mutant embryos. Moreover, in mouse LMTK⁻ cells transfected by the 5-HT_{2B} cDNA, 5-HT raises the endogenous expression but not the phosphorylation levels of ErbB-2 protein (data not shown). Furthermore, the 5-HT_{2B} receptor expression remains unchanged in ErbB-2 knockout mice (22). We also investigated if ablation of 5-HT_{2B} receptor selectively reduced expression of other genes that are involved in heart development. We performed reverse transcription-PCR as a quantitative approach for the expression of genes whose ablation resulted in defects in the heart, such as *N-myc* or for chamber-specific markers such as *MLC2v*. No significant changes in either marker (*N-myc* or *MLC2v*) mRNA expression were detected in the 5-HT_{2B} mutant embryos (data not shown). The mechanism whereby 5-HT_{2B} activation regulates ErbB-2 expression is currently under investigation in our lab. Because ErbB-2 but not ErbB-4 receptor levels are impaired in the 5-HT_{2B} mutant mice, our data suggest that Gq-coupled 5-HT_{2B} receptor specifically uses ErbB-2 receptor tyrosine kinase pathways in the heart.

Interestingly, mice lacking both Gαq and Gα11 also die at embryonic day 11 because of hypoplasia in the ventricular wall.

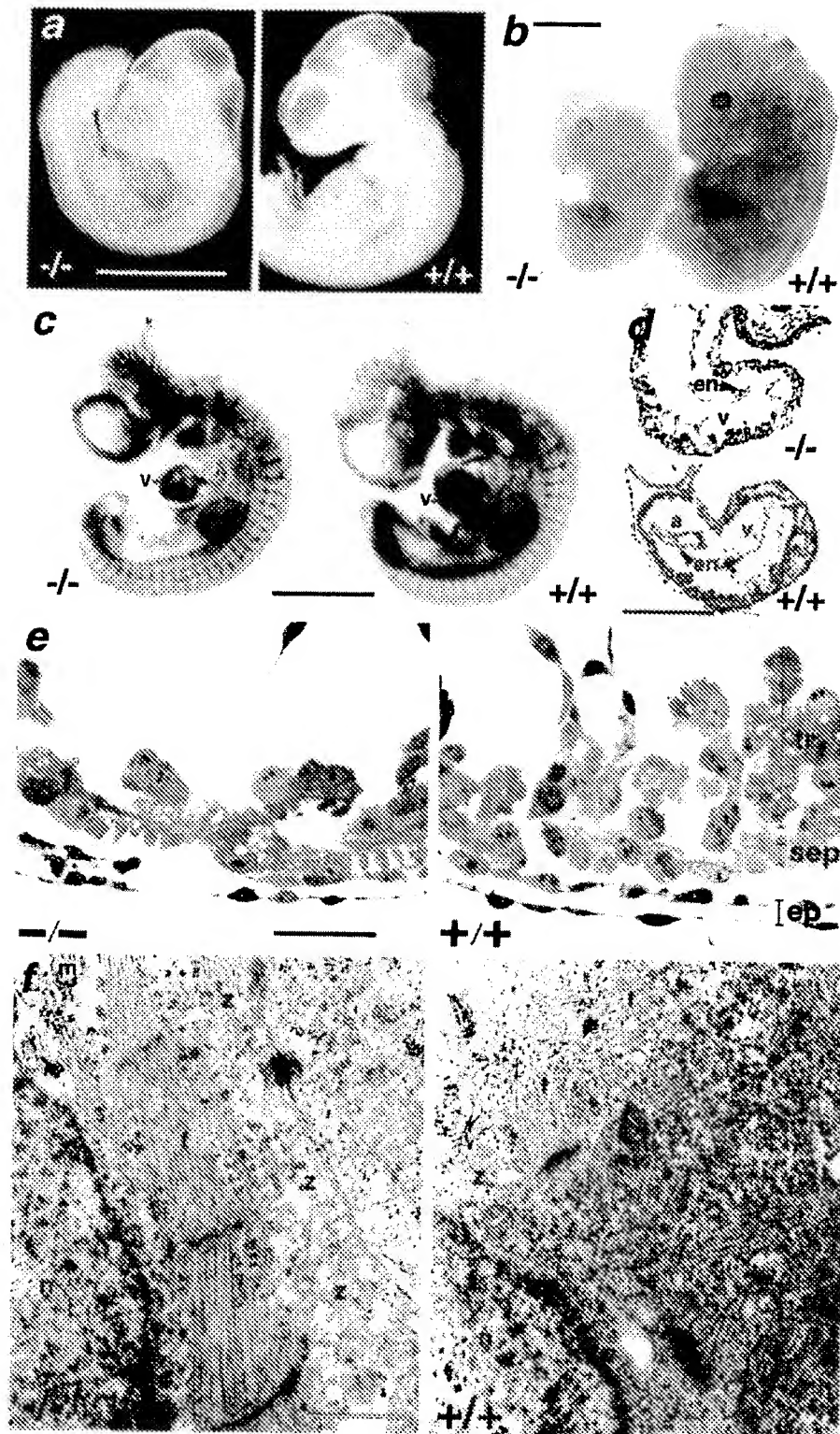


Fig. 2. Morphology of 5-HT_{2B} mutant embryos. (a) 5-HT_{2B}-deficient embryos at 10.5 dpc exhibit a typical bleeding into the pericardial cavity. (b) At 12.5 dpc, the mutant embryos are smaller and paler than their wild-type littermates. (c) Whole-mount immunohistochemical staining of 10.5 dpc embryos for developing blood vessels with platelet endothelial cell adhesion molecule antibody revealed no gross defects in vascular patterning compared with age-matched wild-type littermates but reduced staining in heart ventricle (v). (d) Semithin sagittal sections of 9.5 dpc embryos demonstrate a severe reduction in the thickness of the ventricle (v) including both the compact zone and the trabeculae in 5-HT_{2B}-deficient embryos. a, Atrium; en, endocardium. (e) Higher magnification shows a reduction of trabecular cells (tr) in the mutant heart, whereas elongated cells (white arrowhead) are visible in the compact subepicardial layer (sep). Epicardial cells (ep) are normally developed. (f) TEM analysis of these embryos reveals the abnormal sarcomeric differentiation within the subepicardial layer in all observed mutant heart ($n = 6$) but not in wild-type heart. z, Z band of the sarcomeres; f, actin fibers; m, mitochondria; and n, nucleus. Genotype designations are +/+, wild type; and -/-, homozygous mutant. (Bars for a-c = 500 μ m; d = 100 μ m; e = 5 μ m, and f = 0.5 μ m.)

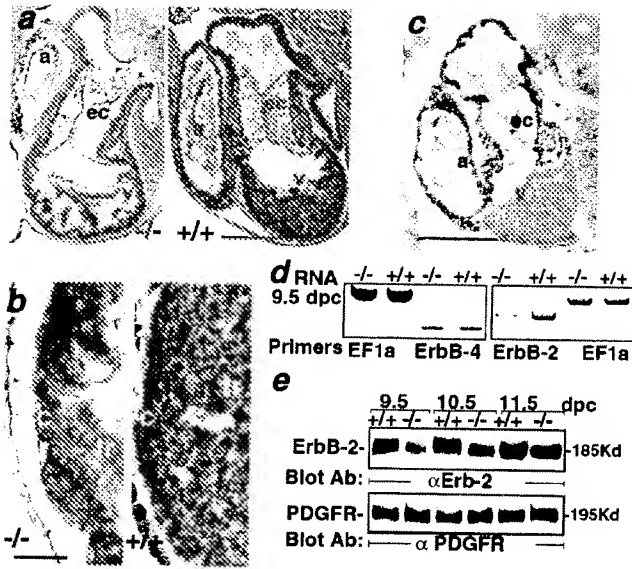


Fig. 3. Reduced ErbB-2 expression in 5-HT_{2B} mutant embryos. (a) The immunohistochemical staining of paraffin sections with anti-ErbB-2 antibody through the heart region of 10.5 dpc wild-type (Right) and mutant embryos (Left). (b) Enlargement of a. The ErbB-2 expression is globally reduced, including over the trabeculae (tr) and the compact zone (c) of the mutant ventricle (v). (c) Immunohistochemical labeling with an anti-5-HT_{2B}-specific antibody performed on cryostat sections of 10.5-dpc embryos illustrates the wild-type expression of 5-HT_{2B} receptor in the trabecular and the compact zone of the heart but not in the endocardial cushions (ec). (d) Semiquantitative reverse transcription-PCR shows a strong reduction of ErbB-2 mRNA in 9.5-dpc mutant embryos whereas ErbB-4 and the ribosomal elongation factor EF1a expression in the same RNA preparation remains unchanged. (e) A Western blot analysis with an anti-ErbB-2 antibody demonstrates a reduction of ErbB-2 expression in total protein extract from 5-HT_{2B} mutant embryos (from 9.5 to 11.5 dpc) (Upper). The platelet-derived growth factor receptor immunoreactivity in the same mutant embryo extracts remains unmodified (Lower). a, Atrium; α, anti-. (Bars for a and c = 250 μm, and b = 25 μm.)

Two active alleles of these genes are required for extrauterine life, and at least one intact *Gαq* allele is needed to bring the embryo to term (23). *Gα11* seems to be preferentially required for 5-HT_{2B}-dependent heart developmental events, because none of the skeletal abnormalities observed in *Gαq*^{-/-}*Gα11*^{+/-} embryos could be detected in the 5-HT_{2B} mutants (data not shown).

The less affected 5-HT_{2B} mutant embryos survived into the neonatal period and appeared normal in their gross morphology although a second period of lethality appears during the first week of life (Fig. 1e). Furthermore, 5-HT_{2B} mutant mice fertility was consistently lower than the control 129/PAS strain, and their body weight was slightly reduced by 9%. Moreover, their percent of heart weight to body weight was significantly reduced by 28% (1.06 ± 0.12 vs. 0.76 ± 0.07 ; $P < 0.05$ by a Fisher test; $n > 5$). Investigation of newborn mutant hearts at histopathological level revealed a strong decrease in ventricular mass and abnormal compact layer in all observed newborn mutant hearts ($n = 6$) (Fig. 4a). Shortly after birth, cardiac myocytes lose their proliferative capacity, and growth of the myocardium occurs through enlargement of existing myocardial cells. A report indicated that 5-HT may regulate proliferation in the embryonic heart (8). We determined the *in vivo* proliferative capacity of myocardium in 5-HT_{2B} newborn heart by measuring BrdUrd incorporation. Essentially, no BrdUrd incorporation was detected in the heart of 5-HT_{2B} mutant newborn (Fig. 4c Left), whereas BrdUrd incorporation was clearly observed in the compact zone of the wild-type newborn heart (Fig. 4c Right). The

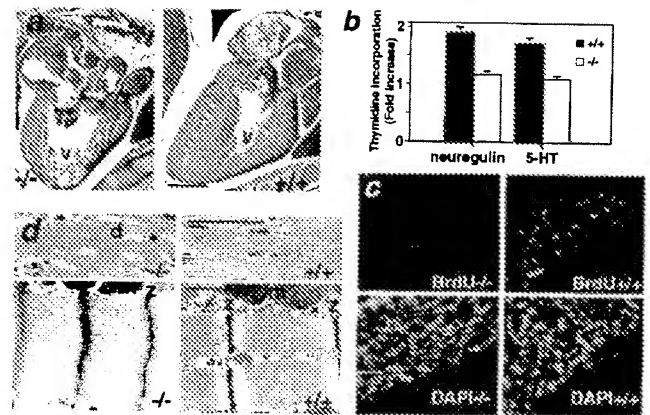


Fig. 4. Heart defects in postnatal 5-HT_{2B} mutant mice. (a) Histological analysis of newborn mutant hearts shows an obvious loss of ventricular (v) mass (hypoplasia). (b) Thymidine incorporation of isolated cardiomyocytes from newborn heart in response to 5-HT and neuregulin (heregulin) is significantly reduced in the 5-HT_{2B} mutant, indicating that myocyte proliferation is impaired and this effect is cell autonomous. The thymidine incorporation rate is expressed as fold increase over basal \pm SEM ($-/-$, $n = 3$; and $+/+$, $n = 3$). (c) *In vivo*, BrdUrd incorporation (green) is almost undetectable in the heart of 5-HT_{2B} newborn mutant (Left), whereas BrdUrd incorporation was observed in the compact zone of control newborn heart (Right). Staining with 4',6-diamidino-2-phenylindole of the same sections localizes the cell nuclei (blue). (d Upper) Semithin sections of heart in 6-week-old mutant shows abnormal morphology, including degenerating fiber (d) (Left). (d Lower) TEM of ultra-thin sections shows that, in mutant heart, the sarcomere lengths are greatly reduced, the M-lines and the I-bands are indistinguishable, and the A-bands occupy the entire length between Z-bands (z) which themselves are thickened (Left) compared with wild-type age-mated heart (Right). m, Mitochondria. (Bars for a = 100 μm; c and d Upper = 20 μm; and d Lower = 0.5 μm.)

hypoplasia in the 5-HT_{2B} mutant heart is probably caused by impaired proliferation.

Accordingly, in isolated newborn ventricular myocytes from 5-HT_{2B} mutant, thymidine incorporation in response to 5-HT was also reduced (Fig. 4b). These *in vitro* data indicate that the 5-HT_{2B} mutant cardiac phenotype is intrinsic to myocytes and is cell autonomous. These data suggest that 5-HT indeed acts as a cardiac mitogenic factor via a 5-HT_{2B} receptor-mediated pathway. Furthermore, the requirement for the neuregulin-ErbB-2 signaling pathways is demonstrated by a thymidine incorporation assay in response to neuregulin (heregulin) in the isolated cardiomyocytes from embryo (9.5 dpc) and newborn mice. Fig. 4b shows that 5-HT_{2B}-deficient cardiomyocytes obtained from newborn hearts also have defective proliferative responses to neuregulin. These data strongly suggest that Gq-coupled 5-HT_{2B} receptor specifically uses the receptor tyrosine kinase ErbB-2 signaling pathways in cardiac development.

Adult hearts from surviving 5-HT_{2B} mutants (6 weeks old) also exhibited dilated chambers and a decreased ventricular mass, their percent of heart weight to body weight was significantly reduced by 24% (0.67 ± 0.4 vs. 0.51 ± 0.03 ; $P < 0.05$ by a Fisher test; $n = 10$). On histological sections, 6-week-old adult hearts from 5-HT_{2B} mutants showed a consistent pattern of myocellular disorganization, and scattered areas of myocellular disarray unlike wild-type hearts (Fig. 4d Upper). At the TEM level, the mutant myocardium displays an irregular array of sarcomeric myofibrils including abnormally wide Z bands relative to controls (Fig. 4d Lower). In particular, the well-defined sarcomeric pattern is disrupted, the sarcomere lengths are greatly reduced, and M bands are indistinct. There were no obvious apoptotic bodies observed at the TEM level in the mutant heart either at 9.5 dpc, newborn, or adult stage. These cardiac sarcomeric defects are similar to those observed in mice

heterozygous for the α -myosin heavy chain knockout (24) with numerous degenerating fibers. Loss of myofibrils is the most obvious structural change in many cardiomyopathies (25) and sarcomeric disarray is characteristic of failing hearts (26). Several studies have suggested a role for Gq-coupled receptors in various heart compartments (27) and Gq has been shown to regulate cardiomyopathy development. For example, both the angiotensin II receptor and α_{1B} -adrenergic receptors signal via Gq. The angiotensin II transgenic mice died *in utero* or shortly after birth with grossly enlarged atria (28), but with no changes in ventricular morphology seen in α_{1B} transgenic mice (29), indicating a specificity of downstream pathways. Parallel to the previous observations, targeted inhibition of Gq signaling by overexpression of a carboxyl-terminal peptide of Gq in the heart reduces ventricular hypertrophy in response to pressure overload (30), whereas D'Angelo *et al.* (31) found that a 4-fold overexpression of the Gq protein in the heart generates cardiac hypertrophy and heart failure.

We show that the 5-HT_{2B} receptor is required for 5-HT to regulate cardiovascular functions. Previously, we have shown that treatment of embryos with selective antagonists of 5-HT_{2B} receptor caused trabeculation defects in the heart along with


defects in neural crest cell derivatives. 5-HT_{2B} mutant mice exhibit obvious cardiac defect but the gross morphology of the CNS appears unaffected. These preliminary observations suggest that the morphological effects of antagonists on neural crest cell derivatives in embryo cultures are probably masked in the 5-HT_{2B} mutant mice, but could be reflected at functional levels. Further investigations are necessary to assess if CNS functions are affected by 5-HT_{2B} receptor elimination. The present data strongly suggest that the Gq-coupled-5-HT_{2B} receptor is involved in cardiac proliferation and differentiation. These findings should facilitate a genetic approach and a new drug design in the cardiovascular disease.

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Ablation of Serotonin 5-HT_{2B} Receptors in Mice Leads to Abnormal Cardiac Structure and Function

Canan G. Nebigil, PharmD, PhD; Pierre Hickel, BS; Nadia Messaddeq, PhD; Jean-Luc Vonesch, PhD; Marie P. Douchet, MD; Laurent Monassier, MD, PhD; Katalin György, PhD; Rachel Matz, PhD; Ramaroson Andriantsitohaina, PhD; Philippe Manivet, PharmD; Jean-Marie Launay, PharmD, PhD; Luc Maroteaux, PhD

Background—Identification of factors regulating myocardial structure and function is important to understand the pathogenesis of heart disease. Because little is known about the molecular mechanism of cardiac functions triggered by serotonin, the link between downstream signaling circuitry of its receptors and the heart physiology is of widespread interest. None of the serotonin receptor (5-HT_{1A}, 5-HT_{1B}, or 5-HT_{2C}) disruptions in mice have resulted in cardiovascular defects. In this study, we examined 5-HT_{2B} receptor–mutant mice to assess the putative role of serotonin in heart structure and function.

Methods and Results—We have generated G_q-coupled 5-HT_{2B} receptor–null mice by homologous recombination. Surviving 5-HT_{2B} receptor–mutant mice exhibit cardiomyopathy with a loss of ventricular mass due to a reduction in number and size of cardiomyocytes. This phenotype is intrinsic to cardiac myocytes. 5-HT_{2B} receptor–mutant ventricles exhibit dilation and abnormal organization of contractile elements, including Z-stripe enlargement and N-cadherin downregulation. Echocardiography and ECG both confirm the presence of left ventricular dilatation and decreased systolic function in the adult 5-HT_{2B} receptor–mutant mice.

Conclusions—Mutation of 5-HT_{2B} receptor leads to a cardiomyopathy without hypertrophy and a disruption of intercalated disks. 5-HT_{2B} receptor is required for cytoskeleton assembly to membrane structures by its regulation of N-cadherin expression. These results constitute, for the first time, strong genetic evidence that serotonin, via the 5-HT_{2B} receptor, regulates cardiac structure and function. (*Circulation*. 2001;103:2973-2979.)

Key Words: cardiomyopathy ■ cell adhesion molecules ■ genetics ■ serotonin

Cardiomyopathy is an important risk factor for subsequent cardiac morbidity and mortality. Relatively little is known about the molecular mechanism underlying cardiomyopathy and heart failure. The neurohormone serotonin (5-hydroxytryptamine, 5-HT) is involved in blood pressure regulation and cardiac function in adults. 5-HT plays an important role in hemodynamic stability. 5-HT-specific reuptake inhibitors (by increasing the availability of 5-HT) produce arrhythmia, including atrial fibrillation, bradycardia, and heart block.¹ The mitogenic action of 5-HT² triggers the valvular fibroplasia observed in carcinoid patients³ and in obese people taking the 5-HT uptake inhibitor/5-HT_{2B} receptor ligand fenfluramine as an appetite suppressant.^{4,5}

The various biological actions of 5-HT are mediated by numerous cognate receptors. There are at least 15 receptor subtypes that belong to 4 classes: 5-HT_{1/5}, 5-HT₂, 5-HT₃, and 5-HT_{4/6/7}.⁶ 5-HT binding to 5-HT_{2A}, 5-HT_{2B}, or 5-HT_{2C} receptors activates phospholipase C, releases inositol trisphosphate, and increases intracellular calcium levels. 5-HT_{2B}

receptor is involved in 5-HT-induced mitogenesis in which c-Src is required for cell cycle progression via the mitogen-activated protein kinase pathway.⁷ Stimulation of the 5-HT_{2B} receptor results in cross talk with the 5-HT_{1B/1D} receptor subtype via activation of phospholipase A₂.⁸ The 5-HT_{2B} receptor also activates nitric oxide synthesis through a PDZ domain.⁹

To understand the specificity of each receptor subtype, the genetic inactivation approach in mice was used. Mutation of 5-HT receptors 5-HT_{1A}, 5-HT_{1B}, or 5-HT_{2C} in mice leads to behavioral abnormalities.¹⁰ We have recently shown that 5-HT_{2B} receptor inactivation in mice leads to trabeculation defects in embryonic heart, causing a 30% lethality at midgestation.¹¹ Now, we investigated cardiopathy in surviving 5-HT_{2B} receptor–mutant mice. This study reveals that 5-HT via the 5-HT_{2B} receptor is involved in the regulation of cardiomyocyte cytoarchitecture and function. 5-HT_{2B} receptor ablation in mice leads to cardiomyopathy, including left ventricular (LV) dysfunction without hypertrophy.

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Methods

Generation of 5-HT_{2B} Receptor-Knockout Mice

Targeted mutagenesis by homologous recombination was described previously.¹¹ All animal experimentation was performed in accordance with institutional guidelines, and protocols were approved by the French Animal Care Committee in accordance with European regulations.

Morphological Analysis of Mouse Embryos

Transmission electron microscopy and histological techniques were performed as previously described.¹² Immunohistochemistry was performed on heart cryosections with the anti-sarcomeric myosin heavy chain (MHC) antibody (MF-20). Anti-tropomyosin and N-cadherin antibody reactions were performed on paraffin sections as described.¹¹ Signal intensity was quantified with a fluorimager (Typhoon, Molecular Dynamics) and calculated as the product of averaged pixel intensity per area.

Cardiomyocyte Density Determination and Confocal Microscopic Analysis

Confocal microscope images of the sections were taken on a Leica TCS4D. Total numbers of nuclei per field were calculated by counting propidium iodide-stained nuclei. Nonmyocytes were tabulated by counting the number of nuclei not surrounded by cytoplasmic myosin, and this number was used to calculate total myocytes as described.¹³

Analysis of Hypertrophic Cardiac Genes by RT-PCR

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed on 1 μ g of total RNA extracted from age-matched control and knockout mice with the ribosomal elongation factor 1A used as an internal control as previously described.¹¹ The following primers were used: atrial natriuretic factor (ANF), 5'-CCAGGCCATATTGGAGCAAA-3' and 5'-GAAGCTGTTGACGCTAGTC-3'; GATA4, 5'-CACTATGGGCACAGCAGTCC-3' and 5'-TTGGAGCTGGCCTGCGATGTC-3'; α -MHC, 5'-CTGCTGGAGAGGTTATTCCTCG-3' and 5'-GGAAGAGTGAGCGGCGCATCAAGG-3'; and β -MHC 5'-TGCAAAGGCTCCAGGTCTGAGGGC-3' and 5'-GCCAACACCACCTGTCCAAGTTC-3'. The PCR products were quantified with an image analyzer (Bio-Rad, GS-700) and calculated as arbitrary units.

Cardiomyocyte Isolation and Video Imaging

Ventricular cardiomyocytes from newborn mice were isolated as previously described.¹⁴ Beating rate in response to dobutamine was determined by video recording of isolated cardiomyocytes. The analysis was performed on the stage of an inverted microscope (Leica DMRiB) with software developed by J.-L.V.

Echocardiographic Methods

Animals (19-week-old mice) anesthetized with sodium pentobarbital (30 mg/kg IP) were observed with 2D-guided M-mode echocardiograms with a short-focal-length, 12-MHz (Hewlett-Packard Medical Systems) transducer. LV end-systolic and end-diastolic diameters (LVESD and LVEDD, respectively) were measured. The percentage of LV fractional shortening was then calculated.

Blood Pressure Measurements

Systolic arterial pressure and heart rate were recorded by the tail-cuff technique with the LE5002 Storage Pressure Meter (Letica) in awake 19-week-old mutant and control mice.

Electrocardiogram

Nineteen-week-old mice anesthetized with tribromoethanol (2.5% solution, 13 μ L/g body wt SC) were recorded with the 4 arms of the ECG leads attached at the origin of each paw by unipolar and bipolar lead derivations. The signal was recorded by an ECG (EKG-Burdick,

Siemens) connected to a data acquisition system (MP100 and Acknowledge Software, Biopac Systems Inc).

Isolated Perfused Heart Preparation

Hearts from mice (12 to 19 weeks old, 23 to 25 g) anesthetized with sodium pentobarbital (60 mg/kg IP) and heparinized (500 U/kg IP) were cannulated and perfused according to Langendorff at 37°C and pH 7.4 with modified Krebs-Henseleit solution containing (mmol/L) NaCl 118, NaHCO₃ 24, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, disodium EDTA 0.5, and glucose 10, gassed with 95% O₂/5% CO₂. Perfusion pressure was constant and equivalent to 100 cm H₂O. The diastolic tension of the suture was adjusted to 1 g during the stabilization period of the heart.

Measurement of Markers for Cardiac Failure and Myocardial Damage

Enzyme immunoassay for creatine kinase-MB isoenzyme and for cardiac troponin I was determined from samples of serum from adult mice.¹⁵

Data Analysis and Statistics

All values represent the average of independent experiments \pm SEM (n=number of experiments as indicated in the text). Comparisons between groups were performed with Student's unpaired *t* test or ANOVA and a Fischer test. Significance was set at *P*<0.05.

Results

Heart Morphology

5-HT_{2B} receptor inactivation leads to partial embryonic death due to trabecular defects in the heart leading to midgestation lethality (30%, n=120).¹¹ The 5-HT_{2B} receptor-mutant mice that reached birth displayed no obvious defects, although 30% (n=120)¹¹ of newborn mice developed signs of fatigue and dyspnea between postnatal days 2 and 5 and died within 24 hours from the onset of these symptoms. A likely cause of neonatal death is inadequate cardiac output due to hypoplasia of the LV, despite the lack of evidence for pulmonary edema. All 5-HT_{2B} receptor-mutant mice that survived the first postnatal week developed to adulthood with cardiac problems. This variation in severity of the phenotype could not be attributed to variability in the genetic background of the mice (all the findings were obtained from 129/PAS pure background mice, and similar mortality was also observed on a C57/Black6J-129/PAS mixed background).

Newborn 5-HT_{2B} receptor-mutant hearts display a striking decrease in the ratio of heart to body weight (28%). This difference was 24% in 6-week-old mutants (Table 1). Histological analysis demonstrated that the decrease in heart mass was restricted to the ventricles (as shown in Figure 1A).

Cardiomyocyte Number and Size

The ratio of cardiomyocytes to total cells (stained with MF-20 antibody, myocyte-specific MHC, and propidium iodide, respectively) revealed 15% fewer cardiomyocytes in the newborn mutants, as shown in Figure 1B. Isolated mutant cardiomyocytes are 12% shorter than wild-type (n>15) (Figure 1C). The decrease in ventricular mass observed in 5-HT_{2B} receptor-mutant mice results, therefore, from decreases in both cell density and size of cardiomyocytes.

TABLE 1. Morphometry of 5-HT_{2B} Receptor-Mutant Mouse Cardiac Parameters

	+/+	-/-
Heart-to-body weight ratio		
Newborn	1.06±0.12	0.76*±0.07
6 weeks	0.67±0.40	0.51*±0.03
Sarcomere length		
Newborn	2.04±0.02	1.38*±0.05
N-Cadherin expression		
Newborn	73.4±12.4	44.0*±8.2
Intercalated disk size		
Newborn	2.54±0.08	0.64*±0.09

Heart-to-body weight ratio is in % (n>5 per group); sarcomere length in μm (n=25 per group, 2 individuals each); N-cadherin expression in arbitrary units (n=10 per group, 4 individuals each); intercalated disk size in μm assessed by direct measurement on electron micrograph per unit picture (n=5 per group, 2 individuals each). Values are expressed as mean±SEM.

* $P<0.05$: difference between mutant (-/-) and wild-type (+/+) mice.

Hypertrophic Gene Expression in Heart

To determine whether the loss of ventricular mass creates compensatory hypertrophic growth associated with altered expression of hypertrophic markers,¹⁶ ANF, α -MHC, β -MHC, and GATA4 expression was evaluated in 12-week-old mutant hearts. Semiquantitative RT-PCR analysis of mutant heart mRNA demonstrated that none of these mRNAs showed significant variation in expression level (<5% variation compared with control, n=5 different individuals). Similar results were obtained in newborn mutants (data not shown).

Cardiomyocyte Function

To determine whether the cardiac phenotype of 5-HT_{2B} receptor-mutant mice was cell-intrinsic, the function of spontaneously beating isolated cardiomyocytes from newborns was investigated. The β -adrenergic receptor agonist dobutamine increased the beating rate of wild-type cardiomyocytes in a dose-dependent manner. Mutant cardiomyocytes, however, exhibited an impaired response to dobutamine in the absence of sympathetic innervation (Figure 2), indicating cell autonomous defects.

Ultrastructural Analysis

A loss of myocardial organization, a scattered area of degenerated cardiomyocytes, and myofibrillar disarray were apparent in newborn mutant hearts. Wavy myofibrils were identified by anti-tropomyosin staining (Figure 3A). In this area, myofilaments appeared misaligned, I bands were not detectable, abnormally wide Z bands were seen, and mitochondria were rounded and irregular (Figure 3B). The sarcomere length in mutants is 33% smaller than that in control mice (n=25). Notably, no evidence for myocardial apoptosis, fibrosis, or significant inflammatory cell infiltrates was found. Nearly identical histopathological findings were observed in all adult mutant hearts.

Furthermore, 5-HT_{2B} receptor-mutant cardiomyocytes had reduced numbers of adherens junctions (Table 1), and the intercalated disks were consistently disorganized (Figure 4A).

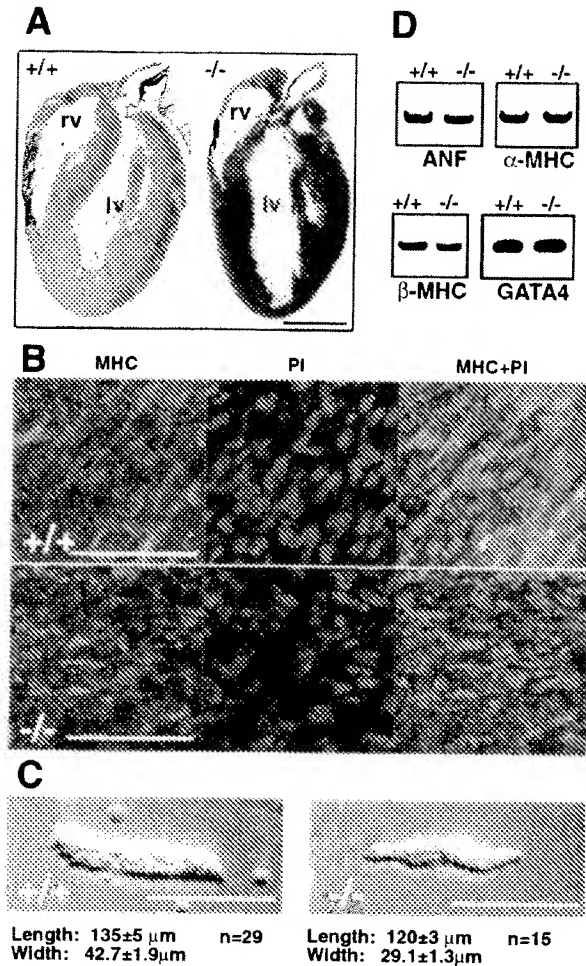


Figure 1. 5-HT_{2B} receptor-mutant mice exhibit decreased ventricular mass due to decreased cell number and size. **A**, Representative sagittal section from paraffin-embedded adult hearts (12 weeks old). rv indicates right ventricle. **B**, Cardiomyocytes (MHC-positive cells) and total cells (propidium iodide (PI)-stained nucleus) per field were counted (n>100 from 4 independent stainings). Numbers are expressed as mean±SD for n=8 sections. **C**, Ventricular myocytes from newborn mice were isolated, and their size was measured. Values are expressed as mean±SEM, n>100. Bars: **A**, 200 μm ; **B**, 50 μm ; **C**, 100 μm . **D**, Markers known to be expressed in hypertrophic growth (ANF, MHC, and GATA4) were analyzed by RT-PCR in adult mice (12 weeks). 5-HT_{2B} receptor-mutant (-/-) and wild-type (+/+) mice.

Z line-associated protein expression was investigated. Vinculin staining in mutant newborn ventricles was unaltered (not shown). N-cadherin expression, however, was reduced by 38.8% in mutant myocardium (Figure 4B, Table 1).

Hemodynamic Measurements

Transthoracic echocardiograms (Figure 5A, Table 2) show LV dilation and reduced systolic performance of the adult mutant mice. In male mutants, the LVEDD was 25% higher than wild-type. Extreme LV dilation (increased LVEDD) was observed, and the LVESD was increased by 50% in male 5-HT_{2B} receptor mutants (n>4). The percent of LV fractional

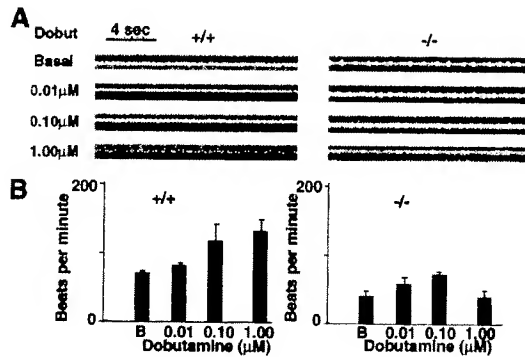


Figure 2. Cardiac phenotype in 5-HT_{2B} receptor-mutant mice is intrinsic to cardiomyocytes. A, Beating rate of isolated cardiomyocytes from newborn hearts in response to different concentrations of dobutamine. B, Beating rate in isolated single cardiomyocytes was deduced from these digital video recordings. Beating rate is expressed as mean myocyte contractions/min \pm SEM. Mutant (-/-, n=5) and wild-type (+/+, n=3) mice.

shortening, as an indicator of systolic cardiac function, was significantly decreased in male (20%) (Figure 5A, Table 2) but not in female mutants (not shown). When myocardial function was measured by Langendorff's heart preparation in vitro, however, the developed force in response to adrenergic stimuli (isoproterenol) was also significantly reduced in female mutants (Table 2, Figure 5B). A slight decrease in mutant female coronary flow was also observed, whereas no apparent change in basal blood pressure or heart rate was detected (Table 2).

ECG Analysis

ECG analysis in mutants revealed neither atrioventricular nor intraventricular conduction defects (similar PR intervals, QRS duration, and amplitude). The resting heart rate was significantly decreased in the anesthetized female mutants. The P duration, but not P amplitude, was significantly

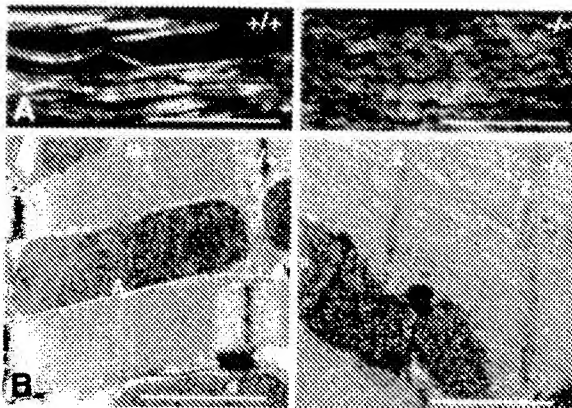


Figure 3. 5-HT_{2B} receptor-mutant mice display dramatic disruption of cardiomyocyte cytoarchitecture with myofibrillar disarrays. A, Representative sections from paraffin-embedded adult hearts stained with tropomyosin antibody. B, High magnification of sarcomeres from LVs. A bands (A), M lines (M), I bands (I), Z bands (Z), desmosomes (d), mitochondria (m), and nexus (n) are shown. Bars: A, 50 μ m; B, 1 μ m. 5-HT_{2B} receptor-mutant (-/-) and control (+/+) mice.

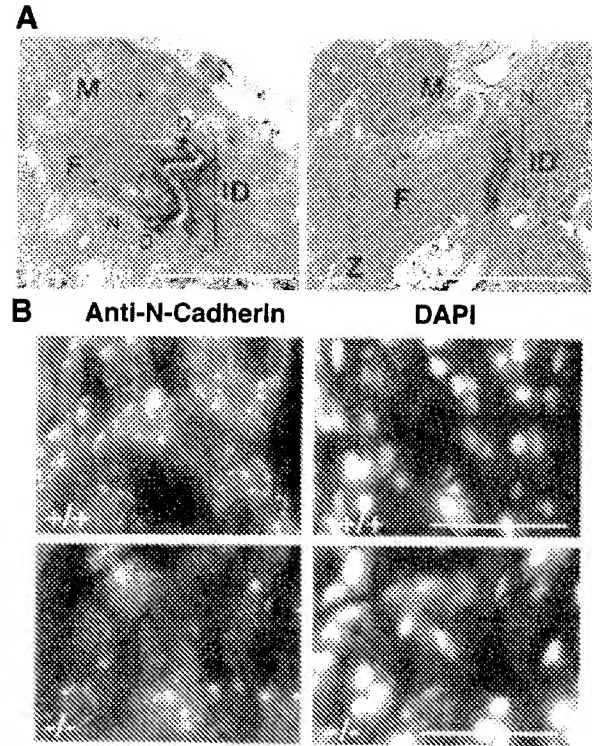


Figure 4. Intracellular junctional structures are perturbed in 5-HT_{2B} receptor-mutant mice. A, High magnification of intercalated disk (ID) structures from LVs of newborn mice. Actin fibers (F), desmosomes (D), mitochondria (M), nexus (N), and Z bands (Z) are shown. B, Immunohistochemistry for intercalated disk protein N-cadherin in newborn hearts. DAPI staining shows distribution of cells (right). Bars: B, 50 μ m; A, 0.5 μ m. 5-HT_{2B} receptor-mutant (-/-) and control (+/+) mice.

increased in female (47%) and to a lesser extent in male (17%) mutants. The most striking difference between wild-type and 5-HT_{2B} receptor mutants (both female and male) was dramatically elevated T-wave amplitude, which is an indicator of abnormalities in repolarization of ventricles (Figure 5C, Table 2). Serum potassium levels, however, were not altered (not shown).

Biochemical Markers of Heart Failure

Clinical indications of human acute myocardial infarction and injury are revealed by serum levels of the cardiac-specific biochemical markers troponin I and creatine kinase-MB.¹⁵ Strikingly elevated markers were observed in the serum of 5-HT_{2B} receptor mutants (6 weeks old) (Figure 5D). Interestingly, male 5-HT_{2B} receptor mutants exhibited more pronounced biological changes than females.

Discussion

In this study, we provide the first evidence that G_q-coupled 5-HT_{2B} receptor ablation in mice leads to cardiomyopathy with LV dysfunction, dilation, and an abnormal structure within the Z band correlated with a deficiency in N-cadherin expression.

5-HT_{2B} receptor-mutant mice exhibit thinning of the ventricular wall and a reduction in ventricular mass that appears

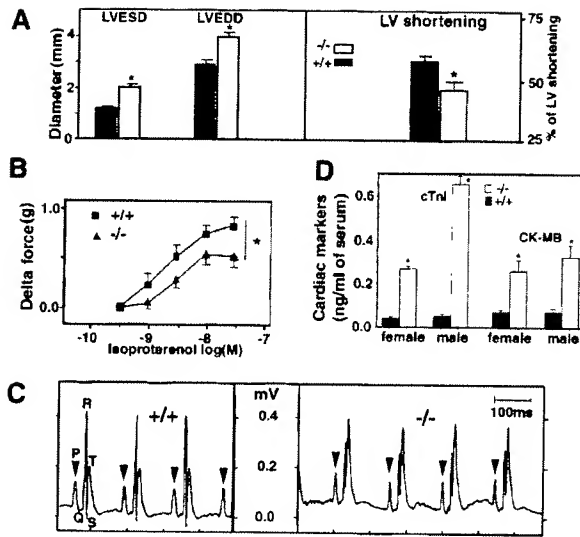


Figure 5. Functional analysis reveals LV dysfunction in 5-HT_{2B} receptor-mutant mice. **A**, Ventricular diameters (left) and LV fractional shortening (right) of adult hearts were obtained by 2D echocardiograms. Values are expressed as mean \pm SEM, $n=5$. **B**, Langendorff preparation of isolated hearts from adult females perfused and stimulated with adrenergic agonist. Values are \pm SEM ($n=3$). **C**, ECG from wild-type (left) and mutant (right) adult males, in 2-arm bipolar derivation. Letters indicate P, Q, R, S, and T waves. Arrows indicate all P waves. **D**, Classic markers of human heart failure, creatine kinase-MB isoenzyme (CK-MB mass measurement) and cardiac troponin I (cTnI), were detected in serum of mice by enzyme immunoassay. Values are ng/mL \pm SEM ($n>10$); *difference between mutant (-/-) and wild-type (+/+) mice ($P<0.05$).

to be due to 2 complementary mechanisms: loss of myocardial cells and decrease in cell size. The primary loss of myocardial cells could be due to apoptosis and/or impaired proliferation of cardiomyocytes. No apoptotic bodies were observed by transmission electron microscopy, yet mitogen-activated protein kinase (MAPK/ERK) activation in response to 5-HT was strongly reduced in newborn mutant hearts (unpublished observations). Together, these data suggest that ventricular hypoplasia is mainly due to impaired proliferation, not to apoptosis. Mutation of the thin-filament protein troponin T in mice also results in cardiomyopathy due to a primary loss of cardiomyocytes and decrease in cell size.¹³ Myofibril loss is the most obvious structural change in human cardiomyopathy,¹⁷ and sarcomeric disarray is characteristic of failing hearts.¹⁸ Actually, the decrease in cardiomyocyte size could be due to impaired growth during postnatal development. The loss of ventricular mass creates biomechanical stress on the remaining viable heart muscle, which typically triggers a hypertrophic response by inducing embryonic gene reexpression. In the 5-HT_{2B} receptor-mutant heart, however, despite increased preload conditions (increased LVEDD), the expression of hypertrophic markers was not elevated, and there were no morphological signs of hypertrophy. Unlike the 5-HT_{2B} receptor-mutant mice, α -MHC- and myf5-mutant mice develop hypertrophy, and interstitial fibrosis accompanied cardiomyopathy.¹⁹ Why 5-HT_{2B} receptor mutants fail to have a hypertrophic response remains to be investigated.

Combined myofibrillar breakdown and inhibited myofibrillogenesis may account for loss of ventricular mass without substantial hypertrophy. Mice overexpressing tropomodulin²⁰ or mutated troponin T are models of dilated cardiomyopathy with inhibited myofibrillogenesis without a hypertrophic response.¹³

Other neurotransmitters and hormones that use G_q protein signaling are also involved in cardiomyopathies. In vitro and in vivo studies have indicated a role for hormones such as angiotensin II, bradykinin B₂,²¹ endothelin 1, norepinephrine, and prostaglandin F_{2 α} , not only in stimulation of cardiac hypertrophy but also in decompensation of the hypertrophied heart through induction of cardiomyocyte apoptosis.²² Targeted expression of the carboxy-terminus of the α -subunit of G_q or overexpression of the G_q protein in the heart causes cardiomyopathy. The regulation of cardiomyocyte cytoarchitecture through the G_q-coupled pathway, however, is poorly understood.

Our data suggest that alteration in cardiomyocyte cytoarchitecture results from 5-HT_{2B} receptor mutation. How does the 5-HT_{2B} receptor affect the organization of myofibrils and related cardiomyocyte cytoarchitecture? 5-HT_{2B} receptor-mutant cardiomyocytes exhibit abnormal organization of contractile elements, including Z-stripe enlargement (Figure 5). Interestingly, most of the mutations leading to dilated cardiomyopathy in humans affect structural proteins involved in cytoskeleton-extracellular matrix interaction at the Z stripe.²³ The altered intercalated disk structures observed in the hearts of 5-HT_{2B} receptor-mutant mice could be a molecular mechanism leading to impaired contractility and myofibrillar degeneration. Z line-associated structures are responsible for the lateral alignment of myofibrils, and their anchorage is at N-cadherin- and vinculin-containing costameres along the cell membrane. The 5-HT_{2B} receptor-mutant mice exhibit decreased N-cadherin levels. N-Cadherin plays an important role in maintaining myofibril integrity,²⁴ in cardiomyocyte interaction, and in myofibrillogenesis.²⁵ Downregulation of N-cadherin and disruption of intercellular adhesion have also been reported in failing guinea pig hearts.²⁶ Addition of antibodies against N-cadherin to cardiomyocyte cultures also induces myofibrillar and cytosolic disorganization.²⁷ Furthermore, mutation of the *Drosophila* 5-HT_{2Dro} receptor (a pharmacological orthologue to 5-HT_{2B} receptor) results in embryos that do not gastrulate properly because of changes in E-cadherin-dependent cell adhesiveness.²⁸ Our data suggest that the 5-HT_{2B} receptor in mammals is required for proper myofibril integrity and myofibrillogenesis by regulating N-cadherin expression.

The 5-HT_{2B} receptor-mutant mouse phenotype has similarity to the natural history of patients with dilated cardiomyopathy. LV dilatation and depressed LV systolic performance in the mutant mice are typical features used to diagnose dilated cardiomyopathy in humans. Moreover, serum biochemical indicators of myocardial infarction are increased in the 5-HT_{2B} receptor-mutant mice. No apparent changes in basal blood pressure and heart rate are detected (Table 2), suggesting that either the 5-HT_{2B} receptor is not involved in basal blood pressure control or systemic vascular flow redistribu-

TABLE 2. 5-HT_{2B} Receptor-Mutant Adult Mouse Cardiovascular Parameters

	Male		Female	
	+/+	-/-	+/+	-/-
Awake animals				
SAP, mm Hg	119±5	121±3	135±3	132±4
Heart rate, bpm	423±18	463±12	446±25	452±16
Isolated heart				
Heart rate, bpm	299±29	272±20	327±25	259±38
Developed force, g	1.94±0.21	1.55±0.33	1.69±0.25	1.41±0.20
Coronary flow, mL/min	1.92±0.20	1.68±0.35	1.83±0.16	1.07±0.20*
ECG				
P, ms	28±2	33±1	19±1	28±3*
P, μ V	112±17	155±31	71±17	79±15
QRS, ms	18±1	16±2	19±2	20±3
PR, ms	56±3	58±2	57±3	64±5
RR, ms	139±61	139±8	123±6	149±7*
QT, ms	71±8	66±5	56±5	56±2
QTc	6±0	6±0	5±0	5±0
T, μ V	228±28	400±40*	211±79	300±68

SAP (systolic arterial pressure) and heart rate were assessed by tail-cuff method on awake animals (n=5 per group); basal heart rate and developed force values were obtained from isolated perfused heart (n=8 per group). ECG was performed on anesthetized animals; QTc=QT/√RR, n=5 per group. Values are expressed as mean±SEM.

*P<0.05: difference between mutant (-/-) and wild-type (+/+) mice.

tion compensates at least partially for this impaired contractility.

5-HT_{2B} receptor-mutant mice exhibit sex differences: Consistent with the idea that the morphological lesions detected in male mutant mice underlie abnormal functions, female mutant mice with less severe histopathological findings did not reveal significant functional changes under steady-state conditions. Similar sex differences occur in other cardiomyopathy models, such as in the β -MHC-mutant mouse.²⁹ In X-linked cardiomyopathy in humans, heart failure occurs rapidly after onset of symptoms in males but is delayed in its onset and progression in females.³⁰ Cardioprotective effects in females have been attributed to estrogen action.

The 5-HT_{2B} receptor-specific agonist norfenfluramine, ergot drugs, and 5-HT released from carcinoid tumors contribute to valvular fibroplasia in humans.^{4,5} The lack of detectable valvular defects in mutant mice, however, indicates that the 5-HT_{2B} receptor is not required for heart valve development.

Mutation of a noncytoskeletal molecule, the 5-HT_{2B} receptor, provides the first genetic evidence that 5-HT, via this receptor, regulates cardiomyocyte function and structure. These findings should facilitate a genetic approach and new avenues of drug design in fighting cardiovascular disease.

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Serotonin is a novel survival factor of cardiomyocytes: mitochondria as a target of 5-HT_{2B}-receptor signaling

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ABSTRACT

Identification of factors regulating cardiomyocyte survival and growth is important to understand the pathogenesis of congenital heart diseases. Little is known about the molecular mechanism of cardiac functions triggered by serotonin. The link between signaling circuitry of external stimuli and the mitochondrial apoptotic machinery is of wide interest in cardiac diseases. Using cultured cardiomyocytes and 5-hydroxytryptamine (5-HT)_{2B}-receptor knockout mice as an animal model of dilated cardiomyopathy, for the first time we show that serotonin via the Gq-coupled 5-HT_{2B}-receptor protect cardiomyocytes against serum deprivation-induced apoptosis as manifested by DNA fragmentation, nuclear chromatin condensation, and TUNEL labeling. Serotonin prevents cytochrome *c* release and caspase-9 and -3 activation after serum deprivation via cross-talks between phosphatidylinositol-3 kinase/Akt and extracellular signal-regulated kinase (ERK) 1/2 signaling pathways. Serotonin binding to 5-HT_{2B}-receptor activates ERK kinases to inhibit Bax expression induced by serum deprivation. Serotonin via phosphatidylinositol-3 kinase/Akt can activate NF- κ B that is required for the regulation of the mitochondrial adenine nucleotide translocator (ANT-1). Parallel to these observations, ultrastructural analysis in the 5-HT_{2B}-receptor knockout mice heart revealed pronounced mitochondrial defects in addition to altered mitochondrial enzyme activities (cytochrome oxidase and succinate dehydrogenase) and ANT-1 and Bax expressions. These findings identify 5-HT as a novel survival factor targeting mitochondria in cardiomyocytes.

Key words: PI3 kinase • Akt • Gq • ERK • Bax

Congenital heart disease is a major cause of disability and morbidity. Relatively little is known about the molecular mechanism of cardiac adaptation (hypertrophy) and maladaptation (apoptosis) underlying cardiac pathogenesis. Several lines of evidence suggest that serotonin [5-hydroxytryptamine (5-HT)] is a neurohormone that regulates cardiovascular functions (1). 5-HT is secreted from enterochromaffin cells into the blood and stored in the platelets. Circulating 5-HT can also be taken up by sympathetic neurons, and vascular endothelial cells and can be coreleased (2). The various biological actions of 5-HT are mediated by numerous cognate receptors. It now appears that there are at least 15 receptor subtypes that belong to four classes: 5-HT_{1/5}, 5-HT₂, 5-HT₃, and 5-HT_{4/6/7} (3). Binding of 5-HT to the Gq-coupled 5-HT_{2A}, 5-HT_{2B}, or 5-HT_{2C} receptors activates phospholipase C (PLC), which initiates a rapid release of inositol trisphosphate and increases intracellular calcium levels. 5-HT_{2B} receptor (5-HT_{2BR}) is involved in 5-HT-induced mitogenesis in which c-Src is required for

cell cycle progression via the mitogen-activated protein kinase (MAPK) pathway (4). Stimulation of the 5-HT_{2B}R results in cross-talk with the 5-HT_{1B/1D} receptor subtype via activation of phospholipase A₂ (5). The 5-HT_{2B}R also activates nitric oxide synthesis through a PDZ domain (6). We have recently shown that inactivation of the Gq-coupled 5-HT_{2B}R gene leads to partial embryonic and neonatal lethality due to the following defects in the heart: 1) 5-HT_{2B}R knockout embryos exhibit a lack of trabeculae leading to mid-gestation lethality. 2) Newborn 5-HT_{2B}R knockout mice exhibit cardiac dilation resulting from contractility deficits and structural deficits at the intercellular junctions between cardiomyocytes. 3) In adult 5-HT_{2B}R knockout mice, echocardiography and electrocardiography confirm the presence of dilated cardiomyopathy (7).

In cultured cardiomyocytes and transgenic animal models, overexpression of Gq-coupled receptors or their signaling molecules, Gq, PLC, or p38 MAPK triggers a hypertrophic response and/or extensive hypertrophy that leads to cardiomyocyte apoptosis (8, 9). On the other hand, several lines of evidence showed that circulating or locally released catecholamine and adenosine via their Gq-coupled receptors contribute to adaptive responses against hemodynamic stress or myocardial injury. Adenosine A₃ receptor activation limits myocardial injury in the isolated rat heart and improves survival in isolated cardiomyocytes, possibly by anti-apoptotic and anti-necrotic mechanisms (10). Stimulation of α -adrenergic receptors activates calcineurin leading to cardiac hypertrophy that protects against ischemia-reperfusion-induced cell death (11). Activation of α -adrenergic receptors inhibits β -adrenergic receptor-induced apoptosis (12). α -Adrenergic receptor activation also protects cardiomyocytes against hypoxia and serum deprivation-induced apoptosis by regulating the expression of mitochondria-associated apoptosis regulatory genes and activating hypertrophic growth (13). These reports indicated that activation of Gq signaling is important for protecting the heart against various stresses. However, the molecular mechanisms involved are not known.

Mitochondria are an important component of the apoptotic signaling (14), especially in the heart (15). In vitro apoptotic signals such as serum deprivation provoke release of cytochrome *c* from mitochondria to the cytoplasm. Cytochrome *c* binds and activates Apaf-1, which in turn activates caspase-9 resulting in the culminating activation of caspase-3 (16, 17) that cleaves key substrates during the apoptotic process (18). Several complex signal transduction pathways have been implicated in the execution of cardiomyocyte apoptosis, including Ras, Raf, MAPK, (19), phosphatidylinositol-3 kinase (PI3K), and protein kinase B/Akt (20). Recently, the Bcl-2 gene family was shown as the central player of apoptosis regulation (21). Some members, such as Bcl-2 and Bcl-XL, inhibit apoptosis, whereas others, such as Bax, Bad, and Bak, accelerate apoptosis by altering mitochondrial membrane permeability thereby inducing cytochrome *c* release. The link between signaling circuitry of external stimuli and the mitochondrial apoptotic machinery is of wide interest in cardiac diseases.

Although ablation of the Gq-coupled 5-HT_{2B}R in mice leads to dilated cardiomyopathy (7), the functional role of 5-HT_{2B}R in heart remains undefined. In this study, for the first time, we identified a cytoprotective 5-HT_{2B}R signaling linking membrane to mitochondrial apoptotic machinery using in vitro and in vivo models.

MATERIALS AND METHODS

Materials

5-HT, PD-09059, and SB-206553 were purchased from R&D Systems. SB-203580 and LY-294002 were purchased from Promega. Phospho-p44/p42 MAPK, phospho-Akt, Akt, phospho-I κ B, and I κ B (I κ B) α -antibodies were purchased from Cell Signaling Technology (Beverly, MA). Mouse MF-20 anti-myosin heavy chain (MHC) antibody was purchased from Hybridoma Bank Laboratories. Anti-cytochrome *c* antibody was from BD PharMingen. All other antibodies were purchased from Santa Cruz Biotechnology. Hoechst and Apoptag TUNEL detection kits were from Boehringer Mannheim. All other reagents were purchased from Sigma.

Generation of 5-HT_{2B}R knockout mice

Targeted mutagenesis by homologous recombination has been described previously (22). All animal experimentation was performed in accordance with institutional guidelines and the French Animal Care Committee in accordance with European regulations approved protocols.

Cardiomyocyte isolation and transfection

Ventricular cardiomyocytes from neonatal mice (3-5 days old) were isolated by Percoll gradient technique as described previously (23). More than 95% of cells exhibited specific MHC-positive cardiomyocytes staining. Cardiomyocytes were grown on plates precoated with fibronectin (Biocoat) in the medium (Dulbecco's + Ham's F-12 medium) containing 10% FCS and 5% horse serum for overnight, and cytosine arabinoside (10 μ M) was added to prevent proliferation of noncardiomyocytes. The cells showed spontaneous contractility within 24 h after plating. For TUNEL analysis and for immunocytofluorescence experiments, cardiomyocytes were plated at a density of 10^5 cells per Lab-TekTM glass slide chamber that was fibronectin coated. For Western analyses, cardiomyocytes were plated at a density of 2×10^6 cells per 35 mm fibronectin coated plastic culture dish.

For MAPK downregulation, cells were treated with mouse p42 and p44^{MAPK} sense or antisense oligonucleotides (30 μ M) during 48 h, and then the medium was replaced by serum free medium (Dulbecco's medium) for 24 h as described previously (4). Fluorescent-labeled synthetic phosphorothioate oligodeoxynucleotides that include the ATG initiation codon of mouse p42 and p44^{MAPK} mRNA, antisense (5'-GCCGCCGCCGCCGCAT-3') or sense (5'-ATGGCGGCGGCGGCGGC-3') oligodeoxyribonucleotides were previously selected and tested for their efficiency (24). The efficiency of transfection was verified by Western blot and fluorescent microscopic analysis revealing that p42 and p44^{MAPK} protein levels were reduced 80% in antisense oligonucleotide treated cells when compared with control or the sense.

DNA laddering

Visualization of apoptotic DNA fragments was performed as described (25). Briefly, after harvesting, cells were treated with lysis buffer (10 mM Tris-HCl, pH 8, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K) and incubated at 50°C for 4 h. After DNA extraction, the purified DNA fragments were labeled with [³²P]ATP and separated by electrophoresis using a 2% agarose gel. The dried gel was exposed to Kodak X-Omat film.

Apoptosis determination by TUNEL analysis

TUNEL analysis of fragmented DNA was performed according to the protocol of the manufacturer as described before (26). Cells were treated with either agonists or antagonist for 4-6 days in serum free conditions. After 6 day of serum deprivation, no induction of endogenous anti-apoptotic factors was observed in cardiomyocytes. Cells then were fixed in 4% formaldehyde and permeabilized. After being washed, slides were incubated with TdT terminal transferase and fluorescein-dUTP. Slides were counterstained with anti-MHC antibody and Hoechst. Cells were scored for TUNEL-positive nuclei corresponding to condensed Hoechst stained nucleus. The percentage of TUNEL-positive cells was evaluated by viewing each field at x60 magnification. Generally, 10 different microscopic fields containing 10-15 cells each were recorded for each sample. Each experiment was repeated at least three times.

Cardiomyocyte virus infection

The adenovirus construct encoding dominant-negative Akt (d3A-Akt) with a K179A/T308A/S473A mutation has been described before (27) and used on isolated cardiomyocytes. The adenovirus construct sIB encoding the I κ B superactive form S32A/S36A was used as described before (28). Cardiomyocytes were plated in medium containing 10% FCS and 5% horse serum overnight and then incubated with adenovirus vector at a multiplicity of infection of 35 in medium containing 2% FCS. After the overnight incubation, the virus was removed and cells were cultured in serum free medium. Infection efficiency was analyzed by GFP signaling using Adeno-GFP-infected cardiomyocytes and is consistently >80% by this method.

Extracellular signal-regulated kinase and Akt and I κ B activity assay and Western blot analysis

MAPK activities were assayed by using phospho-p42/p44 MAPK [extracellular signal-regulated kinase (ERK)1/2] antibodies (4). Stimulated cardiomyocytes were harvested in SDS sample buffer at various time points. Approximately 20 μ g of protein were separated on 10% SDS/PAGE and blotted to nitrocellulose membranes. Two identical blots were incubated with antibody specific for the dually phosphorylated, activated forms of ERK1 and ERK2 and an antibody specific for ERK2 that is independent of its phosphorylation state. Similar Western blot analysis was performed using appropriate antibody for phospho-p38 or phospho-Akt or phospho-I κ B- α . Loading homogeneity was verified by stripping and reprobing the blots. Blots were stripped with 6.25 mM Tris, pH 7.5, 2% SDS, and 100 mM 2-mercaptoethanol for 30 min at 45°C, washed for 1 h, and reprobed with antisera specific for p38 or Akt or I κ B antibodies.

Wild-type and knockout cardiomyocytes were treated with serum free medium and ± 1 μ M 5-HT or ± 5 nM leukemia inhibitory factor (LIF) for 4-6 days and then extracted and submitted to Western blot analyses to determine the cleavage of procaspase-3 and procaspase-9 as a indicator of relative activities using anti caspase-3 and caspase-9 antibodies. Cardiomyocytes were treated with serum free medium and ± 1 μ M 5-HT and ± 10 μ M LY-294002 or 50 μ M PD-098059 for the indicated times and then extracted and submitted to Western blot analyses to determine the relative quantities of Bax and adenine nucleotide translocator (ANT-1) expression. Antibody-antigen complexes were detected with ECL kit according to the instructions of the manufacturer. Densitometric analysis was carried out using Molecular Dynamics Image Quant software.

NF- κ B luciferase assays

Cells were transfected with plasmid coding for a luciferase driven by a minimal TK promoter upstream NF- κ B responsive element. Twenty-four hours after plating, cardiomyocytes were transfected with a plasmid pGL3-NFKB-RE-tk-luc mock (empty vector) using transferrin transfection in combination with Fugene or Lipofectamine according to the recommendation of the manufacturer (4). With this protocol, transfection efficiency in cardiomyocytes was measured by cotransfection of a β -galactosidase-containing construct. Cells were stimulated with different concentration of 5-HT or LIF (5 nM) for 24 h. After cell lysis and removal of cell debris by centrifugation, 150 μ l samples of cell lysate were combined with 50 μ l of luciferase buffer (25 mM Tris, pH 7.8, 1 mM DTT, 15 mM MgSO₄, 4 mM EDTA, 45 mM KHPO₄, pH 7.8, 0.3 mM luciferin, 5% glycerol, 3 mM ATP, and 270 μ M CoA). An MGM Instruments Optolamp II luminometer was used to measure light emission of each sample for 5 s (29).

Analysis of cytosolic and mitochondrial fraction of cytochrome *c*

For cytosolic and mitochondrial fraction of cytochrome *c*, after cardiomyocyte treatment, cells were permeabilized, sampled on 12% SDS gel, and processed for immunoblotting as described by Ekert et al. (30). In brief, cardiomyocytes were plated then suspended in 200 μ l of 0.025% digitonin (Calbiochem) in a lysis buffer (250 mM sucrose, 20 mM HEPES, pH 7.4, 5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mg/ml aprotinin, and 10 mg/ml leupeptin). After 10 min, the cells were centrifuged (2 min, 13 000 rpm) and the supernatant was removed (cytosolic fraction). The remaining pellet was resuspended in 200 μ l mitochondrial lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% Triton-X-100, and 50 mM Tris-HCl, pH 8.0), lysis was allowed to proceed for a further 30 min, and the pellet was centrifuged. Twenty-eight microliters of each fraction was sampled on 12% SDS-PAGE. Densitometric analysis was performed with image analyzer (Bio-Rad, GS-700).

Immunostaining

Immunohistological detection was performed on isolated cardiomyocytes with antiserum against the anti-MHC antibody (MF-20), the cytochrome *c*, ANT-1, and Bax as described previously (7, 22). Signal intensity was quantified on digitalized images and calculated as the product of averaged pixel intensity per area. Densitometric analysis was carried out using Molecular Dynamics Image Quant software.

Morphological analysis of mouse heart

Transmission electron microscopy and histological techniques were performed as described previously (7, 22). For histochemistry, cryostat sections of heart (7 μ M) were fixed and blocked. The standard techniques were used for the immunohistochemistry.

Mitochondrial enzyme activity assay

The histochemical enzymatic test for succinate dehydrogenase and cytochrome *c* oxidase activities was performed on cryostat sections of unfixed heart from wild-type and knockout mice as described (31).

Data analysis and statistics

All values are average of independent experiments \pm SE (n =number of experiments as indicated in the text). Comparisons between groups were performed using an ANOVA followed by a Student's *t* test. Significance was set at $P < 0.05$.

RESULTS

5-HT via 5-HT_{2B}R inhibits apoptosis induced by serum deprivation in isolated cardiomyocytes

To evaluate the effect of 5-HT on cardiomyocyte survival, cardiomyocytes from neonatal heart were isolated and apoptosis was induced by serum deprivation. Apoptosis was detected first by monitoring internucleosomal cleavage with DNA laddering. DNA fragmentation was observed after the day 2 of serum deprivation and persisted throughout day 6. Stimulation by 5-HT (1 μ M) or neuregulin (NRG-1, 25 ng/ml) in the serum free condition protected wild-type but not 5-HT_{2B}R knockout cardiomyocytes from apoptosis as manifested with DNA fragmentation (Fig. 1A). To further confirm the role of 5-HT_{2B}R in protecting cardiomyocyte from serum deprivation-induced apoptosis, we performed both TUNEL and Hoechst staining. As shown on Fig. 1B, apoptotic cardiomyocytes exhibited small condensed nuclei detected by Hoechst staining (blue) corresponding to the TUNEL staining (green). A quantitative analysis revealed that cardiomyocytes subjected to serum free medium for 4-6 days displayed an approximate 38% increase in apoptosis (Fig. 1C). In the presence of 5-HT or NRG-1 (not shown) for 4-6 days, the number of TUNEL-positive cardiomyocytes declined to $8 \pm 5\%$. In the presence of the specific 5-HT_{2B}R inhibitor SB-206553 (1 μ M), 5-HT was not able to inhibit apoptosis (Fig. 1C). Conversely, the 5-HT_{2B}R knockout cardiomyocytes exhibited an approximate 45% apoptotic cells 4-6 days after serum deprivation and still displayed $46 \pm 5\%$ apoptotic cells in the presence of 5-HT (Fig. 1C). These data indicate that 5-HT via 5-HT_{2B}R protects cardiomyocytes from apoptosis.

PI3K/Akt and ERK1/2 activities cross-talks in the anti-apoptotic pathway of 5-HT

To investigate the potential roles of the p38, Akt, or ERK pathways in 5-HT-mediated cytoprotection, cardiomyocytes were incubated with various cell-permeable inhibitors of these pathways in the presence and absence of 5-HT (1 μ M). Under these conditions, PD-098059 (50 μ M), a specific inhibitor of MEK1/2, and thus ERK (32), compromised the ability of 5-HT to protect the cardiomyocytes, resulting in about two times more apoptotic cells than observed in cells treated with 5-HT alone (from 8% in presence of 5-HT to 16% in presence of 5-HT plus PD-098059). Moreover, MAPK downregulation by transfecting cells with MAPK (ERK1/ERK2) antisense oligonucleotides blocked the 5-HT cytoprotective effect to nearly the same extent as seen in the PD-098059 treated cells. LY-294002 (10 μ M), a specific inhibitor of PI3K, and thus Akt (33), also reduced the cytoprotective effects of 5-HT, resulting in about three times more apoptotic cells than observed in cells treated with 5-HT alone (24% of apoptotic cells) (Fig. 2). Accordingly, a dominant-negative d3A-Akt with K179A/T308A/S473A mutations reversed the 5-HT-cytoprotective effect observed with LY-294002 at a similar efficiency. p38 inhibition by SB-203580 (10 μ M) had no effect on the 5-HT_{2B}R-dependent cytoprotective effect of 5-HT in the wild-type cardiomyocytes (data not shown). 5-HT-mediated cytoprotective effect was completely reversed in the presence of both PD-098059 and LY-294002. These results show that

the cytoprotective effects conferred by 5-HT against serum deprivation-induced apoptosis are not only dependent on ERK but also PI3K/Akt pathways.

Since inhibition of the PI3K/Akt and ERK pathways compromised the cytoprotective effects of 5-HT, the ability of the 5-HT to activate these pathways was evaluated using antibodies specific for each kinase at the residues that are phosphorylated upon activation. The relative level of phospho-ERK1/2 was time dependent and reached a maximum value of about twofold over control after 10 min of exposure to 1 μ M 5-HT without altering total ERK-2 level (Fig. 3A). Phosphorylation of ERK1/2 by 5-HT was blocked by 50 μ M PD-098059 (Fig. 3A-C). In 5-HT_{2B}R knockout cardiomyocytes, 5-HT was not able to activate ERK1/2. However, LIF (5 nM), a known survival factor still phosphorylates ERK1/2 in the 5-HT_{2B}R knockout cardiomyocytes. The levels of phospho-Akt (ser 473 and thr 308) were maximal after 15 min of exposure to 5-HT and amounted to about threefold over control (Fig. 3B). Phosphorylation of Akt was blocked by 10 μ M LY-294002 but not by 50 μ M PD-098059 without altering total Akt expression (Fig. 3C). Both ERK and Akt were transiently activated by 5-HT or LIF. Moreover, no p38 phosphorylation in response to 5-HT could be evidenced (not shown), confirming that p38 activation is not involved in 5-HT-dependent cytoprotection. These results are consistent with roles for ERK1/2 and Akt signaling pathways in 5-HT_{2B}R-mediated cytoprotection against serum deprivation-induced apoptosis.

5-HT via 5-HT_{2B}R activates the I κ B - α /NF- κ B signaling pathway

To investigate the downstream regulators of ERK1/2 and PI3K/Akt kinase in cardiomyocytes, the effect of 5-HT on the activation of NF- κ B was assessed using NF- κ B/luciferase responsive element reporter gene. 5-HT stimulated NF- κ B (Rel A)-dependent reporter transcription in wild-type cardiomyocytes, while in 5-HT_{2B}R knockout cardiomyocytes LIF but not 5-HT induced NF- κ B activity (Fig. 4A). NF- κ B staining in cardiomyocytes, initially localized in the cytoplasm in a punctuate pattern, was strikingly increased in the nucleus after 15 min of 5-HT treatment (Fig. 4B). Subsequently, I κ B- α phosphorylation in the presence of 5-HT was determined. 5-HT phosphorylated I κ B- α , reaching a maximum at 15 min (data not shown). Indeed, the phosphorylated form of I κ B- α undergoes gradual degradation at 15 min (Fig. 4C). The time for nuclear translocation of NF- κ B by 5-HT is consistent with the I κ B- α degradation (Fig. 4B). This phosphorylation of I κ B- α by 5-HT was completely inhibited by the PI3K inhibitor LY-294002 but not by the MAPK inhibitor PD-098059 (Fig. 4C). These results show that in cardiomyocytes 5-HT via PI3K/Akt induces I κ B- α degradation thereby NF- κ B nuclear translocation, which activates NF- κ B -dependent gene transcription.

5-HT via 5-HT_{2B}R prevents cytochrome *c* redistribution and caspase cleavage in cardiomyocytes

Next, we investigated the targets of 5-HT signaling for protecting cardiomyocytes from apoptosis. In wild-type cardiomyocytes, cytochrome *c* was localized in the mitochondrial fraction and no cytoplasmic cytochrome *c* was observed (Fig. 5A). However, the cytochrome *c* was substantially translocated from mitochondria to cytosol after serum deprivation. The mitochondrial release of cytochrome *c* into the cytoplasmic fraction was blocked after treatment of wild-type cardiomyocytes with 5-HT (1 μ M) and LIF (5 nM). In 5-HT_{2B}R knockout cardiomyocytes, basal cytoplasmic cytochrome *c* was slightly increased, and after serum deprivation, cytoplasmic cytochrome *c* reached to the maximum that was also observed in wild-

type cardiomyocytes. Conversely, 5-HT did not prevent cytochrome *c* translocation from mitochondria to cytosol, whereas LIF totally prevented this translocation in the 5-HT_{2B}R knockout cardiomyocytes (Fig. 5A). The total cytochrome *c* levels in wild-type and 5-HT_{2B}R cardiomyocytes were similar. These data indicate that only 5-HT-mediated survival effects are impaired in 5-HT_{2B}R knockout cardiomyocytes.

Recent studies have demonstrated that release of cytochrome *c* from mitochondria leads to activation of caspase cascade in cardiomyopathic heart (34, 35). We examined the effect of 5-HT on caspase cleavage as an indicator of caspase activity. A significant increase in cleaved caspase-3 and caspase-9 was observed after serum deprivation, whereas the cleavage was negligible after the 5-HT treatment in wild-type cardiomyocytes. No 5-HT-dependent but LIF-dependent inhibition of caspase-3 and caspase-9 cleavage was observed in 5-HT_{2B}R knockout cardiomyocytes (Fig. 5B).

These data indicate that 5-HT via 5-HT_{2B}R prevents cytochrome *c* redistribution from mitochondria, thereby inhibiting caspase activity to protect cardiomyocytes from serum deprivation-induced apoptosis.

5-HT via 5-HT_{2B}R regulates Bax and ANT-1 expression

Next, we investigated how 5-HT cytoprotective signaling prevents cytochrome *c* redistribution from mitochondria. The intrinsic mitochondria-dependent apoptotic pathway in cardiomyocytes is largely dependent on anti-apoptotic and pro-apoptotic members of the Bcl-2 family proteins. Bcl-2 inhibits apoptosis by blocking the release of cytochrome *c* from mitochondria during cellular stress whereas pro-apoptotic member Bax causes cytochrome *c* release (36). We investigated whether 5-HT regulates Bax expression thereby controlling cytochrome *c* release in isolated cardiomyocytes. In the apoptotic conditions, Bax expression increased twofold that was reduced in the presence of 5-HT. The effect of 5-HT was to downregulate Bax expression that was completely prevented by the ERK1/2 inhibitor PD-098059 (Fig. 6A). When the same blot was revealed with anti-phospho-Bad antibody, no significant difference was observed in the Bad level or phosphorylation stage. These effects of 5-HT were completely absent in 5-HT_{2B}R knockout cardiomyocytes. However, inhibition of PI3K by LY-294002 or inhibition of NF- κ B by the adenovirus sIB encoding the I κ B superactive form S32A/S36A did not change 5-HT-mediated regulation of Bax expression (Figs. 5A and 6A). These data indicate that, in the cardiomyocytes, ERK1/2 activation by 5-HT is involved in regulating Bax expression, whereas PI3K/Akt did not alter Bax levels.

Next, we asked if PI3K/NF- κ B signaling regulates also the mitochondrial membrane permeability. ANT-1 is a component of mitochondrial membrane permeability transition pore (37) and the only mitochondrial carrier for ADP and ATP. Since ANT-1 plays an important role in the disturbed cardiomyocyte metabolism in the dilated cardiomyopathy and ANT-1 mutant mice exhibited severe cardiomyopathy (38), we investigated possible regulation of ANT-1 in 5-HT cytoprotective signaling. In the apoptotic conditions, ANT-1 expression was increased in the wild-type cardiomyocytes but was reduced in the presence of 5-HT. Downregulation of ANT-1 by 5-HT was completely inhibited by the PI3K inhibitor LY-294002 but not by the ERK1/2 inhibitor PD-098059 (Fig. 6B). When NF- κ B was blocked with the adenovirus sIB encoding the superactive I κ B, the 5-HT effect on ANT-1 expression was completely inhibited (Fig. 6B).

In vivo immunodetection of Bax and ANT-1 analysis in the frozen sections of the hearts showed that the Bax and ANT-1 levels were increased by 59 ± 5 and $39 \pm 4\%$, respectively, in the knockout mice heart (Fig. 6B). These results were confirmed by RT-PCR analysis (not shown). Our in vivo and in vitro data indicate that 5-HT/5-HT_{2B}R cytoprotective signaling targets mitochondria by regulating Bax and ANT-1 expression and that 5-HT-cytoprotective signaling is impaired in the 5-HT_{2B}R knockout mice heart.

5-HT_{2B}R knockout mice heart demonstrates abnormal mitochondrial structure and functions

Next, we investigated the mitochondrial structure in the 5-HT_{2B}R knockout mice heart. Electron microscopic analysis in neonatal (Fig. 7A) and 6-wk-old (Fig. 7B) 5-HT_{2B}R knockout mice heart revealed pronounced mitochondrial abnormalities such as interrupted inner membrane and swollen cristae. Although damage in mitochondria is a key step leading to programmed cell death, no ultrastructural nuclear fragmentation but myofibrillar breakdown was observed in the 5-HT_{2B}R knockout mice heart. To investigate how these structural abnormalities are reflected in the in vivo functions of mitochondria, enzymatic histochemical staining for cytochrome *c* oxidase (COX-2) and succinate dehydrogenase (SDH) activity was performed. This staining revealed reduced activity of both SDH and COX-2 by 40 ± 5 and $55 \pm 4\%$, respectively, in the 5-HT_{2B}R knockout mice heart (Fig. 7B). These data indicate that secondary to structural defect in mitochondria of 5-HT_{2B}R knockout mice heart, the electron chain transport and oxidative phosphorylation were disturbed as observed in human dilated cardiomyopathy (39, 40).

DISCUSSION

Although a number of signaling pathways that lead to dilated cardiomyopathy and heart failure have been discovered, the factors that mediate distinct forms of cardiac hypertrophy, apoptosis and survival are not yet elucidated. Using cultured cardiomyocytes and 5-HT_{2B}R knockout mice as a model of dilated cardiomyopathy, we demonstrate that 5-HT_{2B}R signaling regulates mitochondrial structure and function thereby controlling apoptosis and myofibrillar organization in the heart.

Suppression of apoptosis by 5-HT

Overexpression of Gq-coupled receptors or Gq protein itself in cardiomyocytes contributes to the development of hypertrophy and/or ultimate decompensation of cardiac hypertrophy leading to apoptosis (8, 41, 42). On the other hand, evidence suggests that hormones such as angiotensin II, endothelin 1, norepinephrine, and prostaglandin F_{2α} via their cardiac Gq-coupled receptors contribute to adaptive responses after hemodynamic stress or myocardial injury (44). However, the mechanism by which Gq-coupled receptors mediate survival effects has not been clearly elucidated. For the first time, our data show that 5-HT can protect cardiomyocytes from apoptosis after serum deprivation. This protective effect is specifically mediated by the Gq-coupled 5-HT_{2B}R: the cytoprotective effect of 5-HT was completely blocked by a specific 5-HT_{2B}R inhibitor in wild-type cardiomyocytes and absent in the 5-HT_{2B}R knockout cardiomyocytes. Previously, neural crest cells have been shown to exhibit apoptosis after the treatment of embryos with a 5-HT_{2B}R antagonist (44), supporting the survival role of 5-HT.

Mechanisms of protective action of 5-HT

5-HT protects cardiomyocytes from apoptosis by preventing of cytochrome *c* redistribution thereby inhibiting caspase-3 and -9 activation. Our data indicate that in the 5-HT anti-apoptotic signaling ERK1/2 and PI3K/Akt cross-talk to regulate cardiomyocyte survival. In the present study, inhibitors of MAPK efficiently blocked the activation of ERK1/2 by 5-HT but did not interfere with the function of PI3K/Akt, indicating that these two signaling pathway are independent of each other. Our observations are consistent with previous findings showing that both MAPK and PI3K/Akt signaling pathways are essential for antiapoptotic action of both interleukin-5 and stem cell factor (45). The downstream targets of MAPK and PI3K/Akt were unclear in cardiomyocytes. For the first time, we show that 5-HT-mediated PI3K/Akt signaling is involved in I κ B α /NF- κ B activation. Previously, it has been shown that PDGF activates Akt that is directly involved in I κ B/NF- κ B activation (46). Akt is also involved in cardiotrophin activation of NF- κ B (p65) and cytoprotection of cardiomyocytes. Our results indicate that Gq-coupled 5-HT_{2B}R activation leads to degradation of I κ B- α and subsequent translocation of NF- κ B (p50) to the nucleus where NF- κ B can regulate anti-apoptotic gene expression.

Mitochondria are targets of 5-HT-mediated cytoprotection

Cytochrome *c* release due to impaired mitochondria has been observed in several models of apoptosis in cardiomyocytes (39, 47); however, the importance of mitochondria as a direct target for factors protecting cardiomyocytes from apoptosis has not been examined yet. Our findings demonstrate that the 5-HT-induced cardiomyocyte cytoprotection involves cross-talks between PI3K and ERK1/2 pathways that lead to the regulation of ANT-1 and Bax expression to protect of mitochondrial membrane permeability and cytochrome *c* release. In human pancreatic cancer cells, MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-XL, and Mcl-1 and promotes survival (48). Although the Bcl-2 family of proteins, at least in part, controls the mitochondrial apoptosis, we demonstrate that only ERK1/2 inhibition can overturn 5-HT-induced down-regulation of Bax in cardiomyocytes. Parallel to these in vitro findings, a robust increased expression of Bax was observed in the 5-HT_{2B}R knockout mice heart. Bax forms membrane pores that control mitochondrial permeability and release cytochrome *c* (49). Bcl-2 and Bcl-XL inhibit formation of these pores; however, no alteration in the expression of these genes could be detected by RT-PCR analysis of the 5-HT_{2B}R knockout mice heart mRNA (data not shown). We observed that Bax expression level was regulated by ERK1/2 activation in the 5-HT cytoprotective signaling, but the mechanism by which ERK1/2 activation controls Bax expression remains to be investigated.

We also observed that PI3K/Akt signaling maintains the integrity of mitochondrial permeability via a mechanism that is distinct from regulating Bcl-2 expression or Bad phosphorylation in cardiomyocytes (50). Moreover, our data show that PI3K/Akt/NF- κ B controls mitochondrial membrane permeability by regulating the ANT-1 expression. ANT-1 is the only mitochondrial transport system for nucleotides, an important link for energy production and accumulation process. It plays an important role in the disturbed myocardial metabolism in the dilated cardiomyopathy. ANT-1 mutant mice exhibited severe cardiomyopathy (38). Impaired ANT-1 function and increased ANT-1 levels were observed in heart tissue from patients with dilated cardiomyopathy (51), and point mutations in the ANT-1 gene have been reported in humans to generate genetic mitochondrial disease (52). ANT-1 overexpression leads to the phenotypic alteration of the apoptosis, i.e., collapsed mitochondrial membrane potential, cytochrome *c*

release, caspase activation, and DNA degradation (53). In the 5-HT_{2B}R knockout mice heart, elevated expression level of ANT-1 is detected, and we present evidence that ANT-1 is a main target of PI3K/Akt/NF- κ B signaling that controls mitochondrial permeability in the cardiomyocytes. Moreover, transgenic mice overexpressing 5-HT_{2B}R in the heart exhibit decreased ANT-1 levels in the heart (unpublished observation). Whether Bax and ANT-1 interact in cardiomyocytes is currently unknown.

Evidence of mitochondrial involvement for 5-HT-mediated cytoprotection in vivo

Mitochondrial dysfunction has been reported in human cardiac diseases including ischemic and nonischemic heart failure, myocardial infarction, arrhythmia, and myocarditis (54). We also observed mitochondrial structural defects in the 5-HT_{2B}R knockout mice heart by electron microscopy analysis. In the knockout mice heart, reduced SDH and COX-2 activities are indicative of altered functions of electron transport complexes II and IV, respectively. Increased lactate plasma levels in knockout mice confirmed this observation (not shown). Decreased oxidative phosphorylation and respiration that lead to lactate production have also been observed in mitochondrial myopathies of human and in other animal models for dilated cardiomyopathy (35).

Although damage in mitochondria is a key step leading to programmed cell death, no typical apoptotic bodies were observed in the 5-HT_{2B}R knockout mice heart despite impaired myofibrillar structure (7). Knockout cardiomyocytes may be in the pre-apoptotic stage long before nuclear events became morphologically manifested in vivo. Activation of caspases by cytochrome *c* in the failing myocardium induces breakdown of contractile proteins, which constitute the basis of impaired systolic ventricular function without inducing nuclear apoptosis (55, 56). Increased troponin I plasma levels in knockout mice confirmed this observation (7). Accordingly, evidence of cytochrome *c* redistribution from mitochondria to cytoplasm and caspase activation without nuclear morphology of apoptosis has also been observed in idiopathic dilated cardiomyopathic in human heart (57). Our in vivo and in vitro data clearly show that 5-HT/5-HT_{2B}R signaling targets mitochondria. Recently, we observed that transgenic mice overexpressing 5-HT_{2B}R in the heart exhibit mitochondrial proliferation and hypertrophy in the ventricular wall (unpublished observations).

Summary

Our data for the first time show that 5-HT binding to 5-HT_{2B}R activates both PI3K/Akt and ERK kinases in cardiomyocytes to protect mitochondrial damage thereby preventing apoptosis (Fig. 8). 5-HT prevents cytochrome *c* release and caspase-9 and -3 activation after serum deprivation by inhibiting ANT-1 and Bax expression via cross-talks between PI3K/Akt and ERK1/2 signaling pathways, respectively. The regulation of ANT-1 expression results from activation of NF- κ B via PI3K/Akt. Using 5-HT_{2B}R knockout mice as a model of dilated cardiomyopathy, we demonstrate that the Gq-coupled 5-HT_{2B}R signaling regulates mitochondrial structure and function (Fig. 8).

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Fig. 1

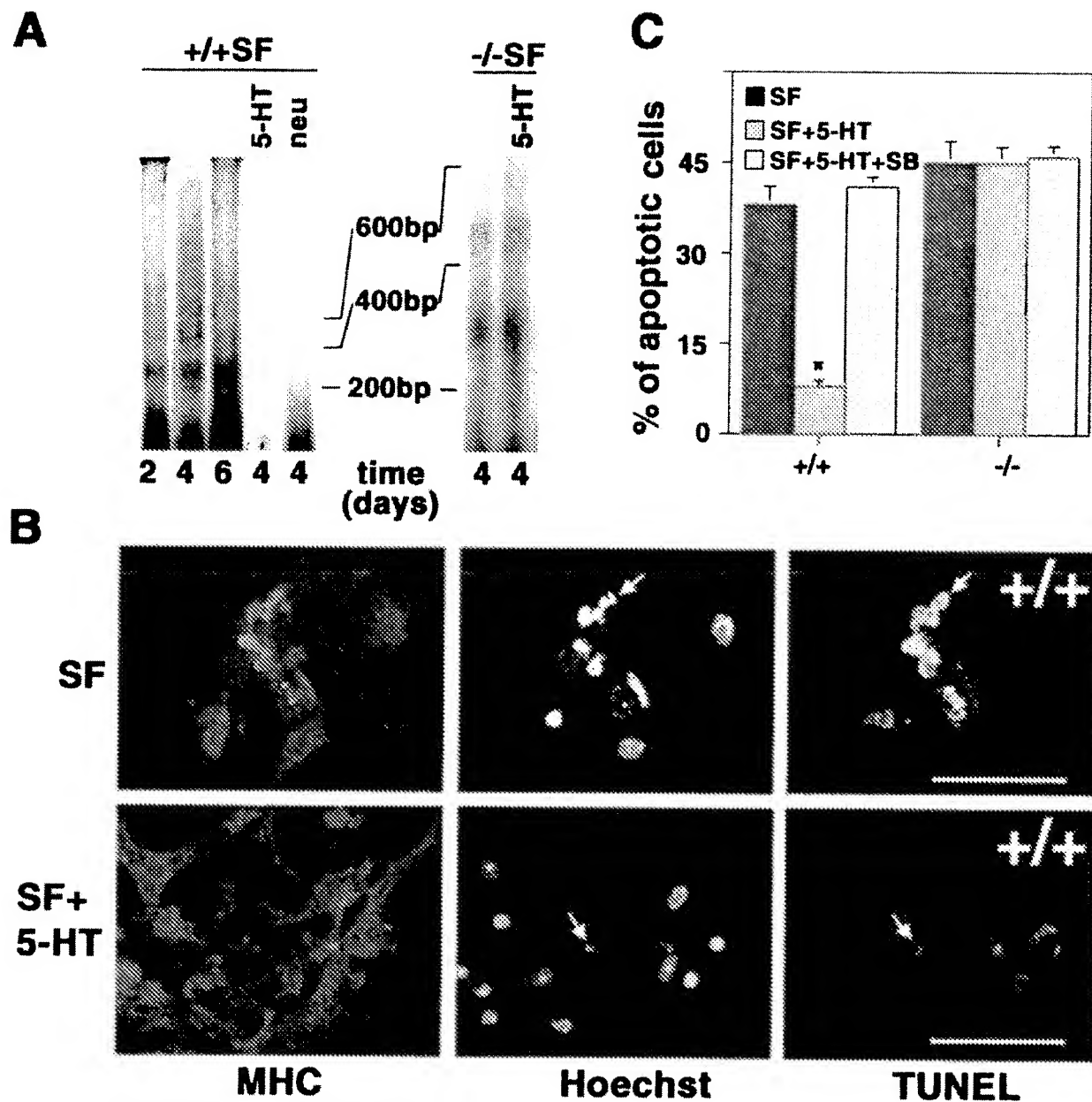


Figure 1. 5-HT via 5-HT_{2B}R acts as a survival factor in cardiomyocytes. **A)** 5-HT prevents DNA laddering induced by serum deprivation in isolated wild-type cardiomyocytes. DNA laddering was observed from 2, 4, to 6 days of serum deprivation (SF). The effect of 5-HT (1 μ M) or neuregulin (NRG-1, 25 ng/ml, neu) was studied by DNA laddering at 4 days of serum deprivation in wild-type (+/+) or knockout cardiomyocytes (-/-). The DNA size marker is in base pairs (bp). **B)** 5-HT prevents apoptosis induced by serum deprivation in wild-type cardiomyocytes. Myocardial cells plated 4-6 days in serum-free (SF) media, \pm 1 μ M 5-HT or \pm 1 μ M SB-206553 (SB), a specific 5-HT_{2B}R receptor antagonist, were then fixed for TUNEL (green) analysis and Hoechst (blue) staining. MHC staining (red) shows the pure population of cultured cardiomyocytes. Illustrations show cardiomyocytes that are representative of the cell population observed following the treatments indicated above. White arrows indicate double-stained, Hoechst- and TUNEL-positive cells with small and condensed nuclei. **C)** Quantitative analysis of TUNEL staining. The number of TUNEL-positive cells in each microscopic field was determined, as described in Methods, and then normalized to the total number of cells in that field to obtain the numbers of TUNEL-positive cells as a percentage of the total. Each value is mean of 10 separate fields (\sim 15 cells/field) \pm SE (* P <0.05). These experiments are representative of 3 similar experiments.

Fig. 2

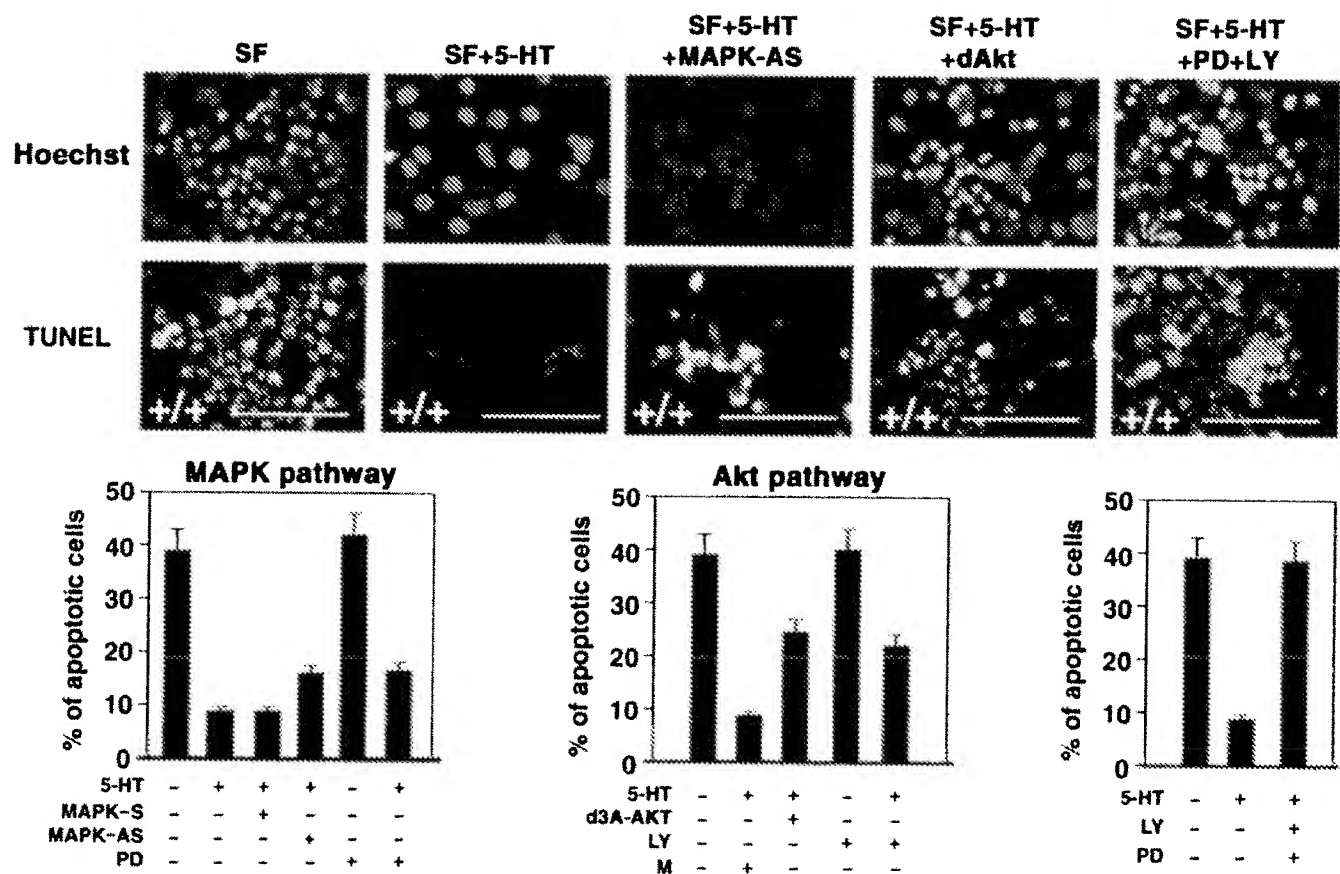


Figure 2. Effects of cell-permeable inhibitors and dominant-negative signaling proteins on 5-HT-mediated inhibition of apoptosis. Illustrations show wild-type cardiomyocytes that are representative of the cell population following treatments inhibiting either MAPK or PI3K pathways. For MAPK inhibition, cardiomyocytes were treated in serum-free conditions with $\pm 1 \mu\text{M}$ 5-HT and $\pm 50 \mu\text{M}$ PD-098059 (PD). For MAPK downregulation, cells were treated with mouse p42 and p44^{MAPK} sense (MAPKS) or antisense (MAPK-AS) fluorescent-labeled synthetic phosphorothioate oligodeoxynucleotides ($30 \mu\text{M}$) during 48 h, then in serum-free medium for 24 h, and then with $\pm 1 \mu\text{M}$ 5-HT for 4 days. Expression levels of each protein were approximately equal, as determined by Western blot analysis (not shown). To inhibit Akt activity, a similar experiment was performed with $\pm 1 \mu\text{M}$ 5-HT and $\pm 10 \mu\text{M}$ LY-294002 (LY). To downregulate Akt, cardiomyocytes were plated in medium containing 10% FCS and 5% horse serum overnight and then incubated with adenovirus construct encoding a dominant-negative Akt (d3A-AKT) with a K179A/T308A/S473A mutation in medium containing 2% FCS. After the overnight incubation, the virus was removed and cells were cultured in serum-free medium. Infection efficiency was evaluated by counting GFP-positive cells using Adeno-GFP-infected cardiomyocytes. Each inhibitor trial was paired with its own control, 6 days after cells were fixed for TUNEL analysis. The number of TUNEL-positive cells in each microscopic field was determined, as described in Methods, and then normalized to the total number of cells in that field to obtain the numbers of TUNEL-positive cells as a percentage of the total. Bars = $500 \mu\text{m}$. Each value is the mean of 10 separate fields (~ 150 cells/field) \pm SE. These experiments are representative of 3 similar experiments. M, mock transfection with the virus.

Fig. 3

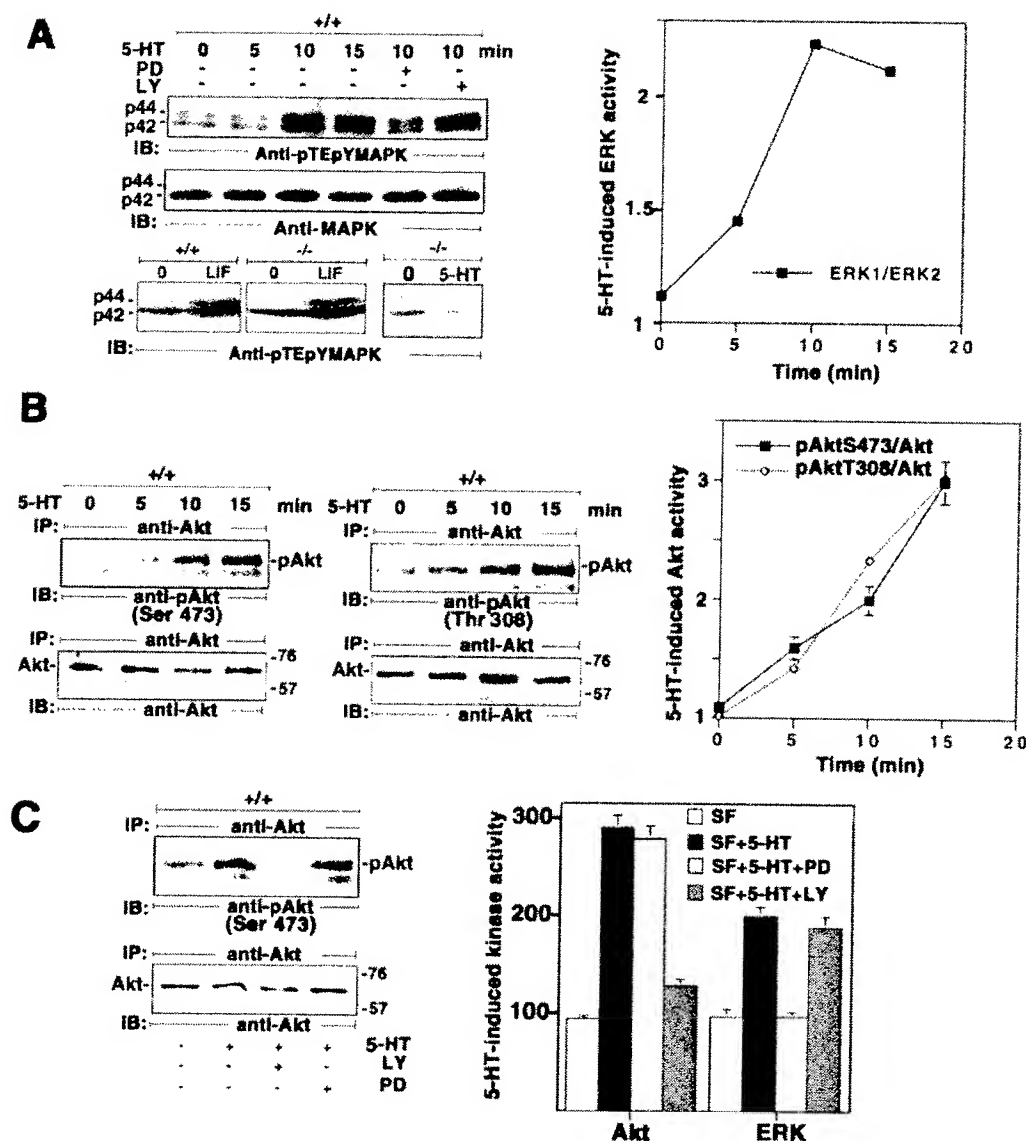


Figure 3. 5-HT activates ERK1/2 and Akt kinases. Cardiomyocytes were treated $\pm 1 \mu\text{M}$ 5-HT and $10 \mu\text{M}$ LY-294002, or $50 \mu\text{M}$ PD-098059 for indicated times, and then extracted and submitted to Western blot analysis to determine the relative quantities of phosphorylated and total, Akt, or ERK, as described in Methods. **A)** 5-HT increases ERK1/2 phosphorylation in a time-dependent manner. As revealed using anti-phospho-MAPK antibody (anti-pMAPK thr 202; tyr 204) (pTEpYMAPK), the ERK1/2 phosphorylation peaks at 10 min in wild-type cardiomyocytes (+/+) but not in knockout cardiomyocytes (-/-). Two identical blots were incubated with antibody specific for the dually phosphorylated, activated forms of ERK1 and ERK2, and an antibody specific for ERK2 that is independent of its phosphorylation state to verify samples homogeneity. ERK activation was evaluated in the presence of 5-HT \pm PD-098059 or \pm LY-294002 and in the presence of LIF in both wild-type and knockout cardiomyocytes. **B)** 5-HT enhances Akt phosphorylation in a time-dependent manner. As revealed with anti phospho-Akt antibodies (anti-pAkt ser 473 or anti-pAkt thr 308), Akt phosphorylation peaks at 15 min in wild-type cardiomyocytes (+/+). 5-HT does not modify the Akt expression as revealed by anti-Akt antibody (anti-Akt) after blot restripping. **C)** Akt activation by 5-HT is completely blocked by LY-294002 but not by PD-098059. Akt activation by 5-HT was evaluated in presence of inhibitors LY-294002 or PD-098059. Quantification of these experiments presented in the graphs was determined using densitometry and Molecular Dynamics ImageQuant software and shown as average SE. IP, immunoprecipitation, IB, immunoblot. Blots are representative of 3 separate experiments.

Fig. 4

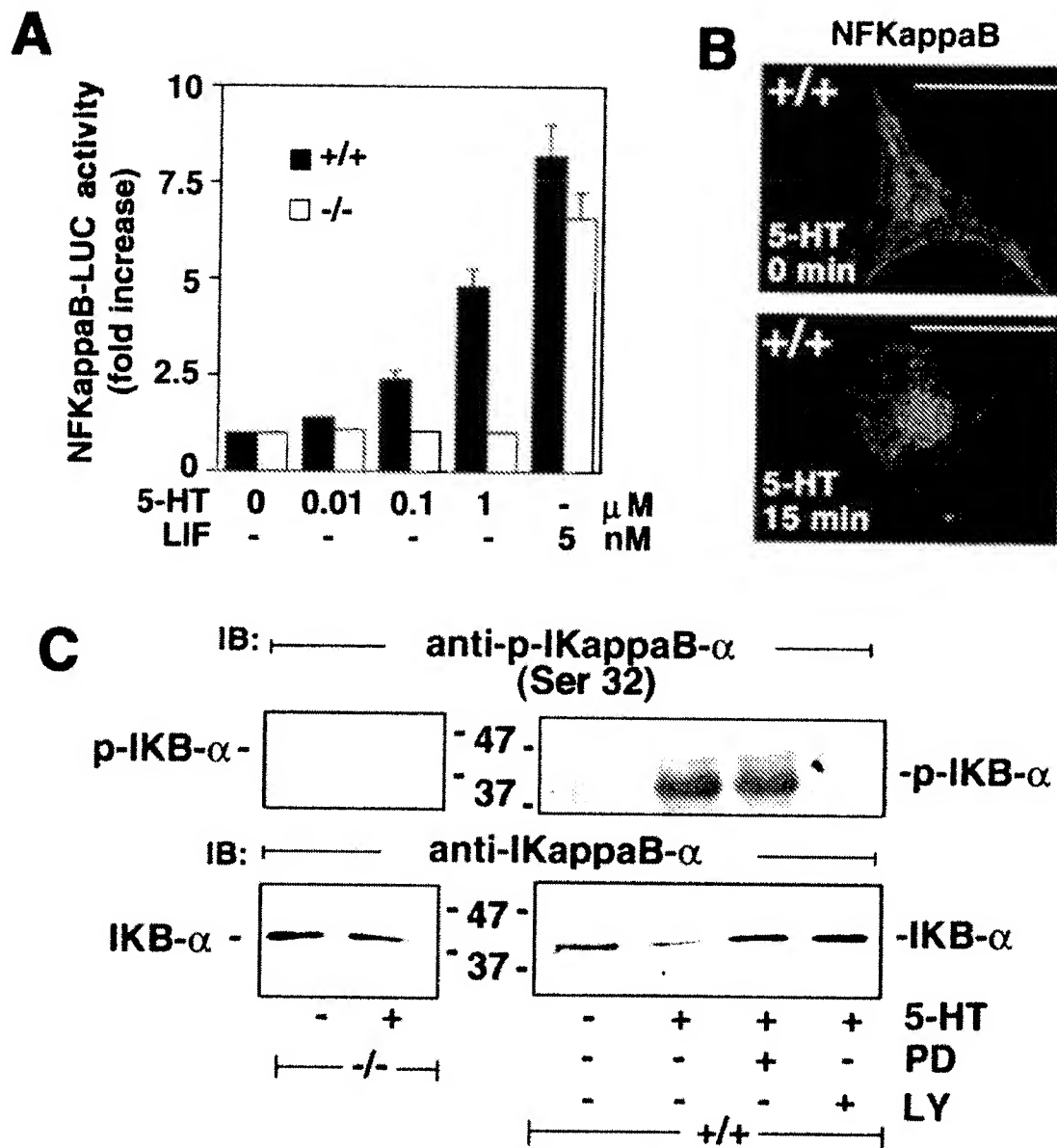


Figure 4. Effects of 5-HT on IKB degradation, NF-KB-mediated transcription, and cellular localization. **A)** 5-HT activates NF-KB-mediated transcription. Wild-type and knockout cardiomyocytes were co-transfected with an (pGL3-NF-KB-RE-tk-luc, 12 μg) and β-galactosidase reporter (20 μg). After 24 h in serum-free medium, the cells were treated for another 24 h with serum-free medium ± 5-HT or LIF at the concentrations indicated and then extracted and assayed for luciferase and β-galactosidase activities as described in Methods. This experiment is representative of 3 similar experiments. **B)** 5-HT induces p50/NF-KB nuclear translocation. Cardiomyocytes were incubated in serum-free media for 24 h, treated ± 1 μM 5-HT for the indicated times and stained for p50/NF-KB (NFKappaB). Primary antibody localization was visualized using CY3-conjugated secondary antibody to show the localization of NF-KB after 5-HT treatment. Shown are single myocytes that are representative of the cell population following the treatments indicated. This experiment is representative of three similar experiments. Bars = 5 μm. **C)** 5-HT-mediated IKB phosphorylation is inhibited by PI3K inhibitor. Cardiomyocytes were treated in serum-free medium with ± 1 μM 5-HT and ± 10 μM LY-294002, or ± 50 μM PD-098059 for 4 days, and then extracted and submitted to Western blot analyses to determine the relative quantities of phosphorylated and total IKB after blot restripping. This experiment is representative of 3 similar experiments.

Fig. 5

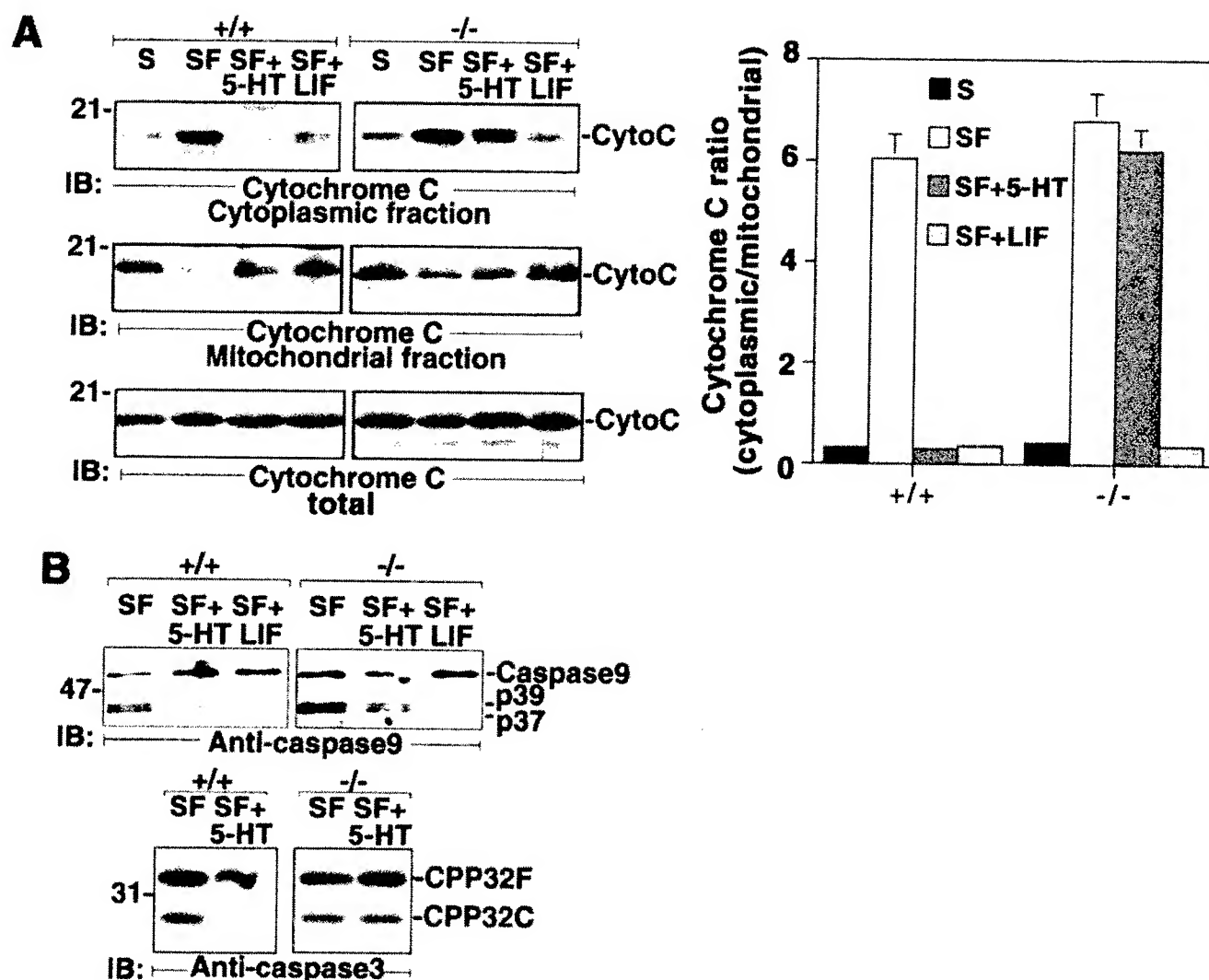


Figure 5. Effects of 5-HT on cytoC cellular localization. A) 5-HT prevents translocation of cytoC from mitochondria to cytosol in wild-type cardiomyocytes. Wild-type and in knockout cardiomyocytes were treated in serum, and serum-free medium with $\pm 1 \mu\text{M}$ 5-HT or $\pm 5 \text{ nM}$ LIF, and then mitochondrial and cytosolic fractions were extracted and submitted to Western blot analyses to determine the relative quantities of cytoC, as shown and as described in Methods. Blots are representative of 3 separate experiments. Values presented in the graphs were determined using densitometry and Molecular Dynamics ImageQuant software and shown as average SE. B) 5-HT prevents caspase activation in wild-type cardiomyocytes. Wild-type and knockout cardiomyocytes were treated in serum-free medium with $\pm 1 \mu\text{M}$ 5-HT or $\pm 5 \text{ nM}$ LIF for 4 days and then extracted and submitted to Western blot analyses to determine the cleavage of procaspase-3 and procaspase-9 as an indicator of relative activities using anti caspase-3 and caspase-9 antibodies. Procaspase-3, CPP32F; cleaved caspase-3, CPP32C. This experiment is representative of 3 similar experiments.

Fig. 6

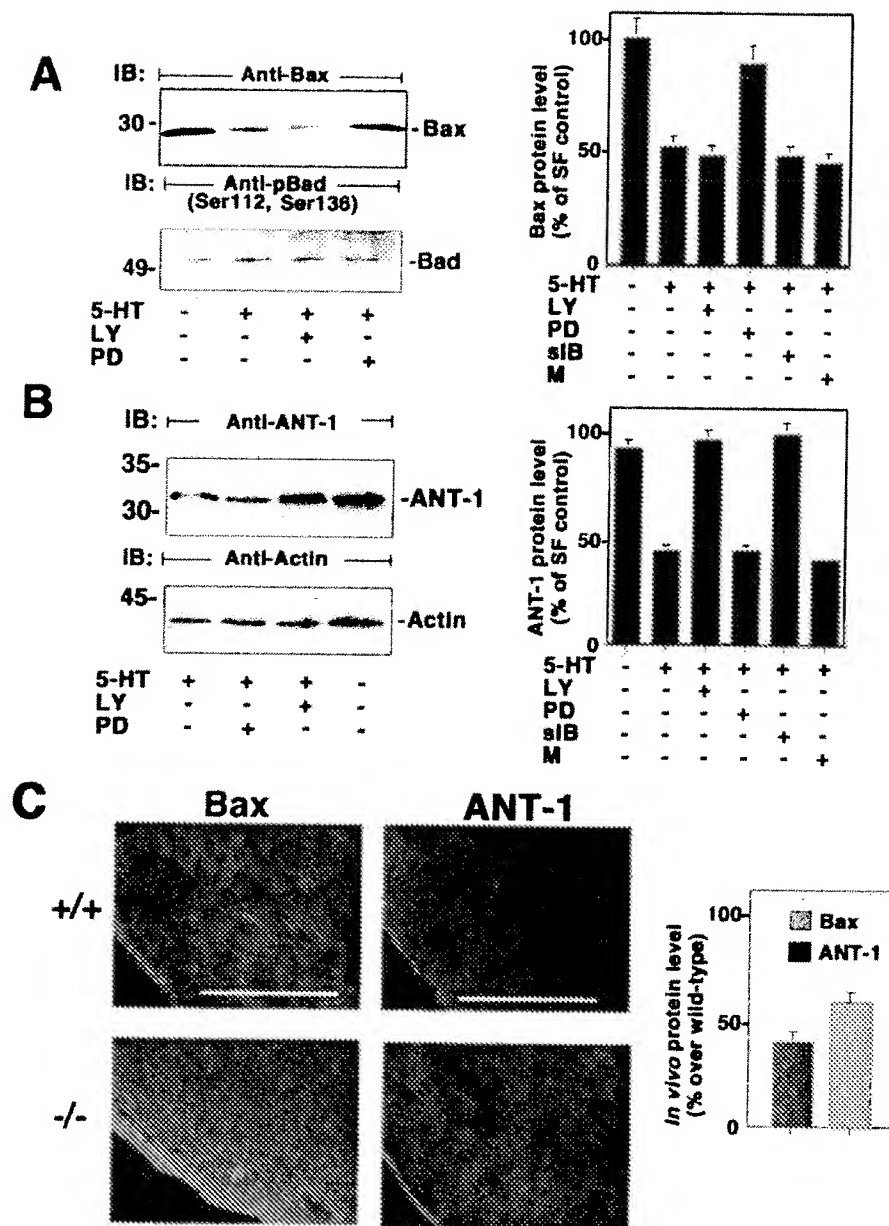


Figure 6. Effects of 5-HT on Bax and ANT-1 levels. A) Reduction of Bax expression by 5-HT. Wild-type cardiomyocytes were treated in serum-free medium with $\pm 1 \mu\text{M}$ 5-HT and $\pm 10 \mu\text{M}$ LY-294002 or $50 \mu\text{M}$ PD-098059 for 4 days and then extracted and submitted to Western blot analyses to determine the relative quantities of Bax, as described in Methods. Phospho-Bad (anti-pBad ser112, ser 136) expression was assessed after restripping of the same blot. Values determined by densitometry using Molecular Dynamics ImageQuant software are shown as a graph \pm SE. B) Reduction of ANT-1 expression by 5-HT. Cardiomyocytes were treated $\pm 1 \mu\text{M}$ 5-HT and $\pm 10 \mu\text{M}$ LY-294002 or $50 \mu\text{M}$ PD-098059 or \pm Adenovirus sIB (sIB) or \pm Adenovirus GFP (M) and then extracted and submitted to Western blot analyses to determine the relative quantities of ANT-1, as described in Methods. Actin expression was assessed after restripping of the same blot. Values determined by densitometry using Molecular Dynamics ImageQuant software are shown as a graph. C) Bax and ANT-1 expression are increased in knockout mice heart. Bax and ANT-1 expression were evaluated by immunohistochemical staining of cryosections of wild-type and knockout mice heart. Bars = $500 \mu\text{m}$. Values of staining intensity determined using Molecular Dynamics ImageQuant software are shown as a graph \pm SE. This experiment is representative of 3 similar experiments.

Fig. 7

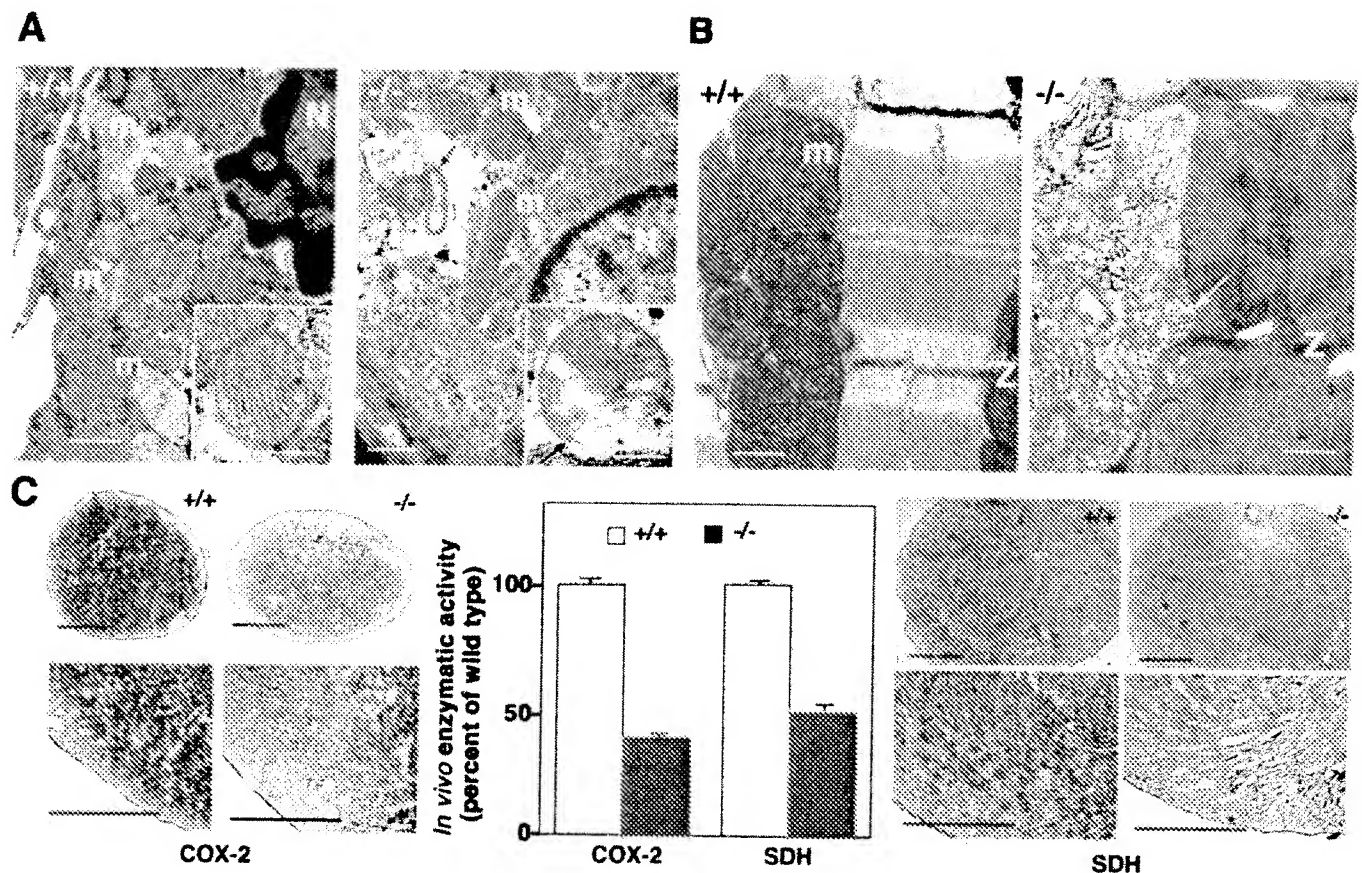


Figure 7. In vivo mitochondrial defects in 5-HT_{2B}R knockout mice heart. **A)** Mitochondrial ultrastructural defects in 5-HT_{2B}R knockout newborn mice heart. The analysis of the compact layer of newborn mice heart was performed by transmission electronic microscopy on ultra-thin sections. Mitochondria (m) with apparent intact morphology in wild-type heart (+/+) and mitochondria with defective membrane (arrow) are indicated in knockout mice heart (-/-). Bars = 0.5 μ m. Insets show higher magnification of the mitochondrial defects. Bars = 0.2 μ m. N, nucleus; Z, z-stripe. **B)** Mitochondrial ultrastructural defects in 5-HT_{2B}R knockout adult mice heart. Analysis by transmission electronic microscopy of ultra-thin sections of 6-wk-old mice heart knockout (-/-) and wild type (+/+) in the compact layer was performed. Fused mitochondria (m) with apparent intact outer membrane (arrow) and defective inner membrane are indicated. Bars = 0.5 μ m. **C)** Decreased mitochondrial enzyme activities in the knockout mice heart. Enzymatic histochemical staining for cytoC oxidase (COX-2, brown) and succinate dehydrogenase (SDH, blue) activity were determined in sections of the heart of wild-type (+/+) and knockout newborn mice (-/-). Bars = 500 μ m. SDH and COX-2 activities in the mice heart are shown as percentage of wild-type values. The values of staining intensity were determined using Molecular Dynamics ImageQuant software and shown as a graph \pm SE. Illustrations are representative of at least 3 separate experiments.

Fig. 8

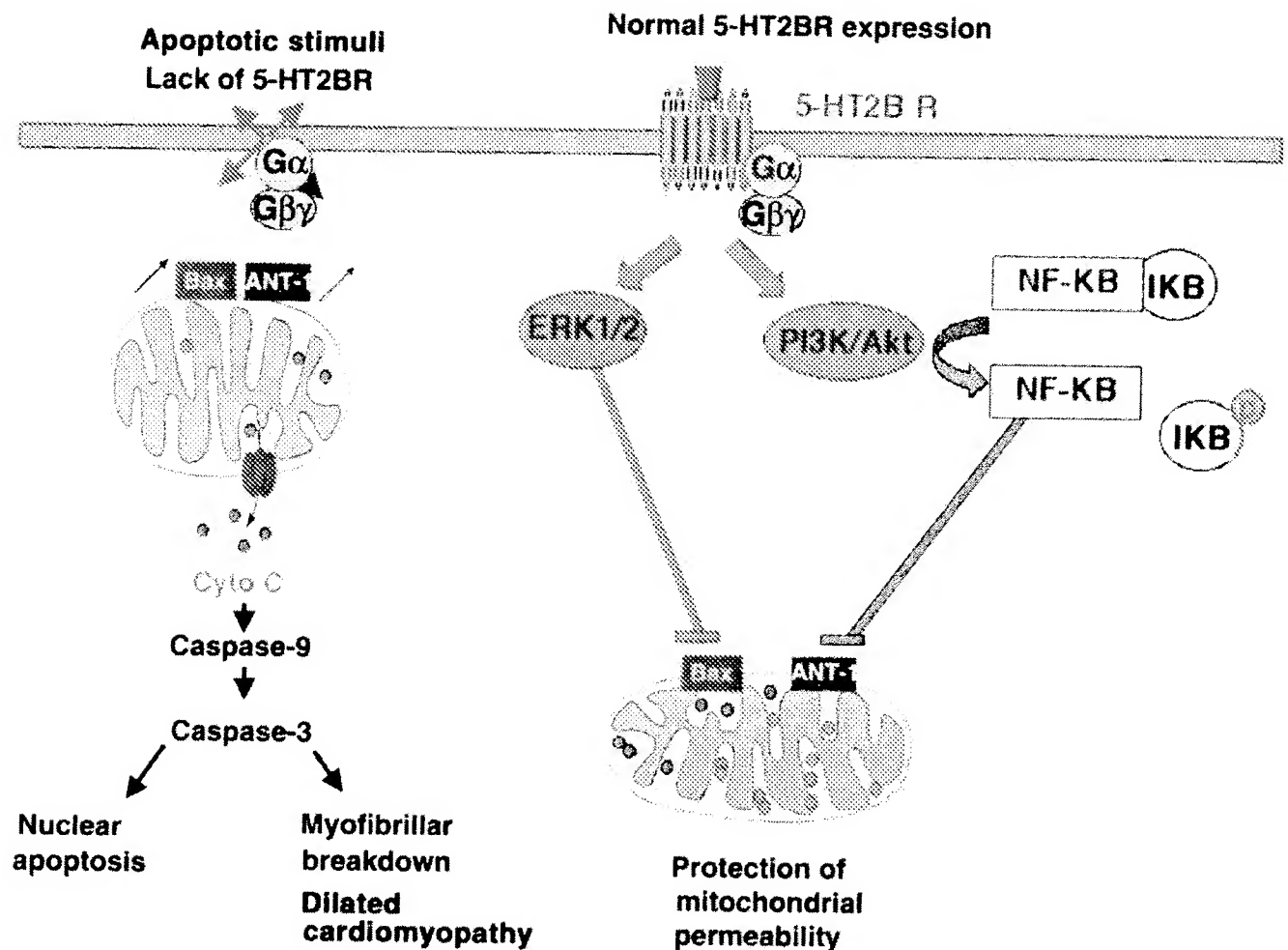


Figure 8. Schematic presentation of 5-HT cytoprotective signaling pathways in cardiomyocytes. 5-HT binding to 5-HT_{2B}R activates both PI3K/Akt and ERK kinases. ERK activation inhibits serum deprivation-induced Bax expression that controls mitochondrial membrane permeability. Parallel to this pathway, upon Akt activation, phosphorylated IκB-alpha triggers the degradation of IκB-alpha to release NF-κB free. This activation of NF-κB by 5-HT inhibits serum deprivation-induced ANT-1 expression to regulate also mitochondrial permeability. A cross talk between PI3K/Akt/ANT-1 and ERK/ Bax pathways targets mitochondria to prevent cytoC release and caspase activation in the 5-HT_{2B}R cytoprotective signaling. In the 5-HT_{2B}R knockout mice as a model of dilated cardiomyopathy, we demonstrate that the Gq-coupled 5-HT_{2B}R signaling regulates mitochondrial structure and function, thereby controlling myofibrillar organization in the heart.

EXHIBIT

11

The effects of antihistamines on cognition and performance

Gary G. Kay, PhD Washington, DC

Allergic diseases are responsible for substantially more disability than is generally realized. Allergic rhinitis alone results in 3.5 million lost workdays and 2 million missed school days in the United States each year. Comorbid conditions such as asthma and sinusitis can be disabling as well, resulting each year in more than 10 million missed school days and more than 73 million days of restricted activity, respectively. Antihistamines continue to be the mainstay of treatment for allergic disorders. In the case of the first-generation antihistamines, however, the treatment may well be worse than the disease. Although these agents are effective H_1 -receptor antagonists, they are also highly lipophilic and readily cross the blood-brain barrier, causing considerable sedation. The second-generation agents are more lipophobic and possess different ionic charges than the first-generation antihistamines. As a result, they are far less likely to cross the blood-brain barrier and, for that reason, cause little if any sedation. In a recent comparative trial, subjects who were treated with the first-generation agent diphenhydramine were found to have significant performance deficits on tests of divided attention, working memory, vigilance, and speed. By contrast, subjects who were treated with the second-generation antihistamine loratadine performed as well as subjects who were treated with placebo. The sedative effects of the first-generation agents persist well into the next day and thus can potentially interfere with daytime performance and safety even when taken the night before. It is therefore recommended that patients whose occupations require vigilance, divided attention, or concentration receive only second-generation antihistamines. (*J Allergy Clin Immunol* 2000;105:S622-7.)

Key words: Allergic rhinitis, antihistamines, functional capacity

Allergic diseases are extraordinarily common in the United States, affecting more than 20% of the population.¹ A recent *Time* magazine article reported that up to 40% of the population may experience allergic rhinitis. Allergic rhinitis alone affects more than 40 million individuals in the United States each year.¹

The everyday misery caused by the sneezing, wheezing, coughing, and itching that accompany allergic diseases is well known. The strong link between allergic rhinitis and such serious conditions as asthma and sinusitis is also understood with increasing clarity.¹ What is generally less recognized is the enormous impact that these conditions have on functional capacity in both adults and children.

Abbreviations used

CI: Confidence interval
OR: Odds ratio

Part of this functional disability is caused by the disease itself. Allergic rhinitis, for example, is known to cause substantial systemic symptoms in addition to its localized symptoms (eg, weakness, malaise, irritability, fatigue, headache, and anorexia).¹ Decrements in cognitive functioning and learning capacity have been demonstrated to occur even in atopic adults and children² independent of medication. It is primarily these systemic effects of allergic rhinitis and the resulting diminished functional capacity that are responsible for the 3.5 million workdays and 2 million school days that are lost each year to allergic rhinitis alone.¹ Corollary conditions such as asthma and sinusitis exact a substantial toll as well; asthma accounts for more than 10 million missed school days each year, and sinusitis results in more than 73 million days of restricted activity annually.¹

Importantly, however, it is not only the disease itself that diminishes mood, energy, and cognitive functioning, but it is also the treatment of the condition. In the United States, sedating antihistamines continue to be the mainstay of treatment for allergic disorders, as they have been for the past 5 decades. Antihistamines are typically categorized as either first-generation agents or second-generation agents (Table I). There are substantial differences between these two classes of antihistamines with respect to their impact on safety, performance, and mood.

EVOLUTION OF THE ANTIHISTAMINES

The first-generation antihistamines were discovered approximately 60 years ago and have been available for at least the last 40 years. These agents are highly potent competitive inhibitors for histamine, acting at the histamine H_1 -receptor site on most target cells in the respiratory mucosa. However, these agents are characterized by ethylamine moieties, which make them highly lipophilic and hence easily able to penetrate the blood-brain barrier and occupy H_1 -receptor sites in the brain, a large number of which are located on the frontal lobes and in the deep structures of the brain. In fact, positron emission tomography studies conducted by Yanai et al³ have demonstrated that the first-generation agents occupy approximately 75% of the H_1 -receptor sites in the brain. The well-known central nervous system side effects of these agents (including fatigue, drowsiness, and performance impairment) are a result of this

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TABLE I. Selected first- and second-generation oral antihistamines

	Onset of Action (h)	Sedative side effects	Possible cardiac side effects
First-generation antihistamines			
Brompheniramine (Dimetapp)	1	Yes	No
Chlorpheniramine maleate (Chlor-Trimeton)	1	Yes	No
Diphenhydramine hydrochloride (Benadryl)	1	Yes	No
Second-generation antihistamines			
Cetirizine (Zyrtec)	1-2	Yes	No
Fexofenadine (Allegra)	1-2	No	No
Fexofenadine/pseudoephedrine (Allegra-D, 12 hr)	1-2	No	No
Loratadine (Claritin)	1-2	No	No
Loratadine/Pseudoephedrine (Claritin-D, 12 h; Claritin-D, 24 h)	1-2	No	No

characteristic. Because of these side effects, the first-generation antihistamines are required to carry a precautionary statement that admonishes users against operating a car or dangerous machinery when taking these agents.

The second-generation antihistamines, which were introduced in the mid-1980s, were designed specifically with the goal of producing agents that would be as effective as the first-generation H_1 -receptor antagonists but without their sedative effects. Several variations on the original agents were introduced to accomplish this goal. The second-generation antihistamines are more lipophilic than their predecessors, and they possess a different ionic charge. In addition, they are made up of larger molecules than the first-generation agents. These 3 factors in combination appear to be what makes it substantially more difficult for these molecules to cross the blood-brain barrier. This is not to say that they do not do so at all; the second-generation agents are capable of passing through to the brain, but they do so to a far lesser extent. Yanai et al³ have shown that the second-generation agents occupy approximately 20% of H_1 -receptor sites in the brain, substantially fewer than do their first-generation predecessors. Furthermore, the level of cognitive dysfunction was shown to correlate with brain-receptor occupancy. Therefore, it is not surprising that the second-generation antihistamines are less sedating than the first-generation antihistamines and that specific agents, namely loratadine and fexofenadine, are not required to carry a warning for sedation and, in fact, exert virtually no sedating effects at their therapeutic dose.

EFFECTS OF SEDATING ANTIHISTAMINES ON RISK OF OCCUPATIONAL INJURIES

Recent research has shown that the warning labels for sedation that appear on the first-generation antihistamines are well deserved and, in fact, should be taken far more seriously than they generally are. In an effort to determine the effect of medication use on work-related injury, Gilmore et al⁴ at the Group Health Cooperative of Puget Sound of Seattle, Washington, conducted a medication-use review of 3394 members of their health maintenance organization

TABLE II. Adjusted ORs of sustaining a work-related injury by class of medication used

Medication used*	OR	95% CI
Narcotic	0.9	0.7-1.1
Nonnarcotic analgesic	1.1	0.9-1.3
Antidepressant	1.2	0.9-1.6
Sedative hypnotic	1.2	0.8-1.7
First-generation antihistamine	1.5	1.1-1.9
Antibiotic	1.2	1.0-1.5
Gastrointestinal medication	1.0	0.8-1.3
Hypoglycemic	1.3	0.9-1.9
Antipsychotic	0.5	0.3-1.1

*Medication use predicated on medication purchase in 30 days before injury date, according to pharmacy records.

Adapted from Gilmore TM, Alexander BH, Mueller BA, Rivara FP. Occupational injuries and medication use. *Am J Ind Med* 1996;30:234-9. Copyright 1996. Reprinted by permission of John Wiley & Sons, Inc.

who had sustained work-related injuries. These cases were compared with data from 2 uninjured matched control groups that were selected from the health maintenance organization membership. The investigators found that the individuals who had used sedating antihistamines had the highest increase in risk of injuries among all of the drug categories studied (including narcotic, nonnarcotic analgesic, antidepressant, sedative hypnotic, antibiotic, gastrointestinal medication, hypoglycemic, and antipsychotic).⁴ Antihistamine use was associated with an increased risk of every kind of injury studied, including open wounds and contusions (odds ratio [OR], 0.5; 95% confidence interval [CI], 11.9), burns (OR, 3.1; 95% CI, 1.0-9.7), and fractures (OR, 1.7; 95% CI, 0.9-3.3). The overall adjusted OR for sustaining a work-related injury was 1.5 for antihistamine users (95% CI, 1.1-1.9) compared with 0.9 for individuals taking narcotics (95% CI, 0.7-1.1; Table II).⁴

EFFECTS OF SEDATING ANTIHISTAMINES ON DRIVING PERFORMANCE

The effects of antihistamines on driving capabilities have been well studied in the Netherlands where "over-

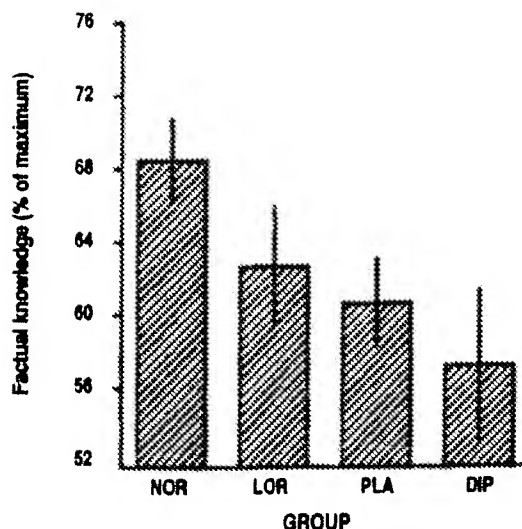


FIG 1. Factual knowledge scores (mean \pm SE) in children with no allergy (NOR) versus children with allergy who were treated with the nonsedating antihistamine loratadine (LOR), placebo (PLA), or the sedating antihistamine diphenhydramine (DIP). (Modified with permission from Vuurman EFPM, van Veggel LMA, Uiterwijk MMC, Leutner D, O'Hanlon JF. Seasonal allergic rhinitis and antihistamine effects on children's learning. *Ann Allergy* 1993;71:121-6. Copyright ACAAI.)

the-road" studies (as opposed to laboratory-simulation driving studies) are often conducted. In one such study, O'Hanlon⁵ compared the tendency to weave while driving in subjects who were being treated with the first-generation antihistamine triprolidine or the second-generation antihistamines terfenadine or loratadine. He found that the subjects who were being treated with either of the second-generation antihistamines demonstrated no increase in the amount of weaving compared with baseline at either 1 or 3 hours after medication ingestion. By contrast, individuals who had taken triprolidine evidenced as much driving impairment as would be expected in an individual with a 0.05 mg/dL blood alcohol level.⁵

These results are consistent with those from an epidemiologic study of automobile fatalities conducted in Ontario, Canada.⁶ This study demonstrated that drivers who were killed in automobile accidents attributed to their own error were 1.5 times more likely to have been using a first-generation antihistamine than were drivers not responsible for the accident in which they were killed.⁶ As a consequence of data like these, 32 states and the District of Columbia have enacted laws against driving while impaired as a result of taking sedating medications, including over-the-counter first-generation antihistamines.

EFFECTS OF ANTIHISTAMINES ON SCHOOL PERFORMANCE IN ATOPIC CHILDREN

Children are also vulnerable to the performance-impairing effects of first-generation antihistamines. Vuurman et al² studied learning performance in a group of 52 primary-school children with a history of seasonal allergic rhinitis. They compared the performance of these

children with that of a group of age-matched children with no allergies. The atopic children received one of three treatments before instruction: a sedating antihistamine (diphenhydramine), a nonsedating antihistamine (loratadine), or a placebo. All of the children returned 2 weeks later, after the allergy season was over, to take tests of their factual knowledge, conceptual knowledge, and ability to apply a learned strategy.

Analysis of the data demonstrated that the atopic children performed consistently less well on measures of factual knowledge ($P < .01$; Fig 1), conceptual knowledge ($P < .02$; Fig 2), and knowledge application ($P < .02$; Fig 3) than did the nonatopic children.² Among the atopic children, however, those treated with loratadine or placebo demonstrated better learning performance than did the diphenhydramine-treated students.² Thus, atopy itself tends to worsen learning capacity in children, and these decrements are exacerbated by treatment with sedating antihistamines. A single-dose treatment with a nonsedating antihistamine was not sufficient to overcome the effects of atopy.

EFFECTS OF FIRST- VERSUS SECOND-GENERATION ANTIHISTAMINES ON SEDATION, COGNITION, MOOD, AND PSYCHOMOTOR PERFORMANCE

In an effort to determine whether the second-generation antihistamines are less likely to cause decrements in the areas of mood, cognition, and sleepiness than their predecessors, our group tested these variables in 98 healthy volunteers who were randomly assigned in double-blind fashion to receive loratadine ($n = 33$), diphenhydramine ($n = 32$), or placebo ($n = 33$).⁷ At baseline, on

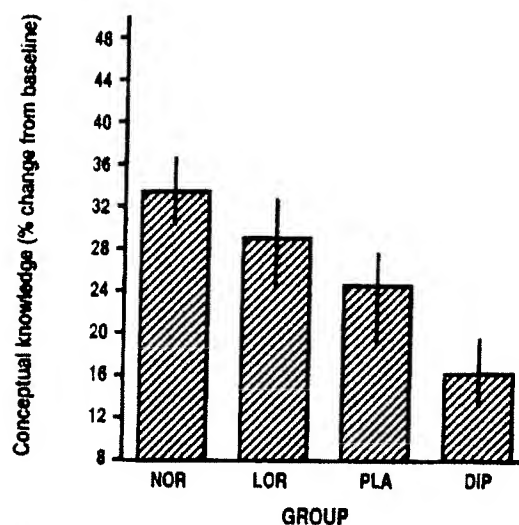


FIG 2. Conceptual knowledge scores (mean \pm SE) in children with no allergy (NOR) versus children with allergy who were treated with the nonsedating antihistamine loratadine (LOR), placebo (PLA), or the sedating antihistamine diphenhydramine (DIP). (Modified with permission from Vuurman EFPM, van Veggel LMA, Uiterwijk MMC, Leutner D, O'Hanlon JF. Seasonal allergic rhinitis and antihistamine effects on children's learning. *Ann Allergy* 1993;71:121-8. Copyright ACAAI.)

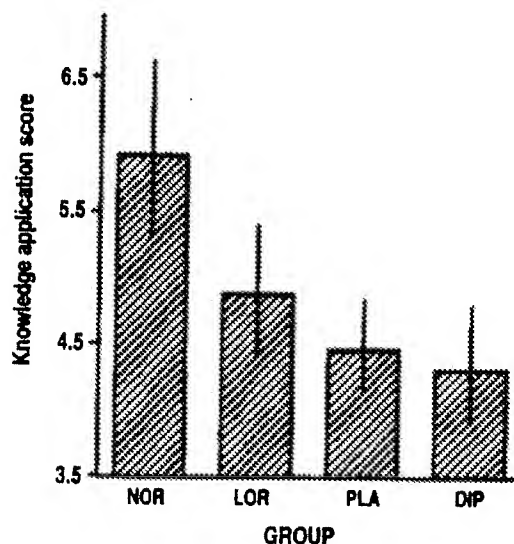


FIG 3. Knowledge application scores (mean \pm SE) in children with no allergy (NOR) versus children with allergy who were treated with the nonsedating antihistamine loratadine (LOR), placebo (PLA), or the sedating antihistamine diphenhydramine (DIP). (Modified with permission from Vuurman EFPM, van Veggel LMA, Uiterwijk MMC, Leutner D, O'Hanlon JF. Seasonal allergic rhinitis and antihistamine effects on children's learning. *Ann Allergy* 1993;71:121-8. Copyright ACAAI.)

day 1 after the administration of the initial dose and on days 3 and 5, these individuals underwent a comprehensive battery of psychometric tests designed specifically to mirror real-world tasks.

On day 1, diphenhydramine (50 mg) was found to have produced substantial adverse effects on divided attention, working memory, vigilance, and speed.⁷ By contrast, subjects being treated with loratadine (10 mg)

performed as well as subjects being treated with placebo. Similarly, on measures of mood, subjects being treated with diphenhydramine reported higher levels of fatigue ($P < .001$) and had lower levels of motivation ($P < .001$) than did subjects being treated with loratadine; they also rated the quality of their test performance as lower ($P < .001$) relative to subjects being treated with loratadine (Fig 4).⁷ Further, subjects being treated with diphenhy-

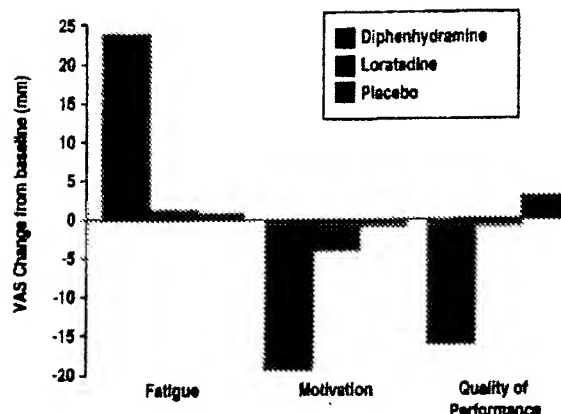


FIG 4. Mean change from baseline in visual analog scale (VAS) ratings on day 1 for subjects who were treated with diphenhydramine, loratadine, and placebo. $P < .001$, loratadine versus diphenhydramine; $P < .001$, placebo versus diphenhydramine. (Adapted from Kay GG, Berman B, Mockoviak SH, Morris CE, Reeves D, Starbuck V, et al. Initial and steady-state effects of diphenhydramine and loratadine on sedation, cognition, mood, and psychomotor performance. *Arch Intern Med* 1997;157:2350-8. Copyright 1997, American Medical Association.)

dramine reported lower levels of activity. The subjects being treated with loratadine, by contrast, evidenced no differences from the subjects being treated with placebo. Although results from testing on days 3 and 5 showed some equilibration among the groups, subjects being treated with diphenhydramine continued to make substantially more tracking errors on tests of divided attention and reported greater fatigue.

Interestingly, in the diphenhydramine group, with those subjects who self-reported that they were not sedated ($n = 22$), performance on measures of divided attention, working memory, and vigilance was substantially impaired. Thus, individuals whose ability to function is compromised by first-generation antihistamines may lack awareness of their reduced level of functioning.

It should be noted that the performance deficits observed in this study are far from inconsequential. In fact, on measures of vigilance, the performance of subjects who were treated with diphenhydramine was approximately a full standard deviation worse than the performance of subjects who were treated with loratadine or placebo. Differences of this magnitude are interpreted by neuropsychologists as indicative of impaired brain function.⁸ Moreover, deficits on tests of attention and vigilance indicate an increased likelihood of errors in the performance of tedious but potentially hazardous tasks of daily living, such as driving an automobile. On similar cognitive tests, differences of the magnitude seen with diphenhydramine are similar to those noted after alcohol intoxication.

EFFECTS OF AN "AM/PM" ANTIHISTAMINE DOSING REGIMEN

The first-generation antihistamines clearly produce sedation and impair performance to a much greater extent than do the second-generation antihistamines.

However, because they are newer, the second-generation agents are associated with higher direct drug costs. Some physicians prescribe a regimen wherein over-the-counter sedating agents are taken at night and nonsedating prescription agents are taken during the day. The rationale behind this so-called "AM/PM dosing regimen" is that patients would "sleep through" the sedative effects of the first-generation agents with no aftereffects the next day.

To test the actual effect of such a regimen on daytime sleepiness and level of alertness, our group studied these variables in 29 healthy volunteers. Subjects were randomized, in double-blind fashion, to one of three parallel treatment groups: an evening dose of 12 mg of the first-generation agent chlorpheniramine plus a morning dose of 60 mg of the second-generation agent terfenadine ($n = 9$); an evening dose of 8 mg of chlorpheniramine plus a morning dose of 60 mg of terfenadine ($n = 9$), or a morning plus an evening placebo dose ($n = 11$).⁹

All subjects underwent the Multiple Sleep Latency Test (an objective physiologic measure of sleepiness) and the Stanford Sleepiness Scale (a measure of subjective sleepiness and alertness). Results from these tests demonstrated that subjects who had received the AM/PM dosing regimens had increased daytime sleepiness and reduced alertness. Subjects who had received evening doses of chlorpheniramine in combination with morning doses of terfenadine fell asleep more quickly during the next day and described themselves as less able to concentrate and less alert than did those subjects who had received placebo. Even after 4 days of the AM/PM regimen, a significantly higher proportion of subjects who were treated with 12 mg of chlorpheniramine had abnormal daytime sleepiness compared with the subjects treated with placebo ($P = .03$). Thus, it is clear that the central nervous system depressant effects of an evening dose of a first-generation antihistamine persist through the next day.⁹

CONCLUSIONS

The first-generation antihistamines negatively affect mood, sleepiness, alertness, and cognitive and psychomotor functioning in adults and children. As a result, these medications can interfere with performance and safety, even when taken the night before. Because patients are frequently unaware of the level to which their performance is impaired, they are likely to fail to use adequate caution in performing potentially dangerous activities, such as driving a car or operating heavy machinery, while under the influence of sedating antihistamines.

Adults who have jobs that demand divided attention, vigilance, and concentration are most likely to be adversely affected by the sedating antihistamines. Adverse effects are not limited to driving and operating dangerous machinery. Performance of clerical and monitoring jobs is also likely to be negatively affected. As a consequence, it is recommended that adults whose occupations demand divided attention, vigilance, or concentration receive nonsedating antihistamines in preference to sedating antihistamines, whenever possible. Children, too, should be treated with nonsedating antihistamines to avoid the adverse effects of the first-generation agents on learning capability.

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EXHIBIT

12

Novel Antipsychotics: Comparison of Weight Gain Liabilities

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Background: We performed a retrospective analysis of 122 clinical records of 92 male patients with DSM-III-R schizophrenia to examine the relative weight gain liabilities of clozapine, risperidone, olanzapine, and sertindole compared with haloperidol. We hypothesized that the unique pharmacodynamic profiles of these agents would contribute to different amounts and patterns of weight gain.

Method: Data were analyzed to determine differences in weight gain during treatment among patients receiving 5 different drug treatments (clozapine [N = 20], olanzapine [N = 13], risperidone [N = 38], haloperidol [N = 43], and sertindole [N = 8]). Measures of maximal weight gain, final weight, and duration to maximal weight gain were calculated.

Results: Repeated measures analyses of variance controlling for age, treatment duration, and initial weight revealed statistically significant differences between groups on all 3 measures. Clozapine and olanzapine had the greatest maximal weight gain liability ($F = 4.13$, $df = 4,23$; $p = .01$). Weight gain with clozapine, but not olanzapine or risperidone, appears to persist (as reflected by final weight) despite behavioral interventions (e.g., nutritional consultation, suggested exercise regimen; $F = 5.69$, $df = 4,23$; $p = .003$). Clozapine- and olanzapine-treated subjects appeared to gain weight over a prolonged period of time, whereas risperidone- and sertindole-treated subjects had a more limited period of weight gain ($F = 2.95$, $df = 4,25$; $p = .04$).

Conclusion: Clozapine and olanzapine caused the most weight gain, risperidone was intermediate, and sertindole had less associated weight gain than haloperidol. The relative receptor affinities of the novel antipsychotics for histamine H_1 appear to be the most robust correlate of these clinical findings.

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Many antipsychotic drugs have been associated with substantial weight gain and drug-induced obesity.¹⁻³ Studies indicate that antipsychotic drug-induced weight gain is a common cause of noncompliance and discontinuance of treatment, resulting in the return of psychotic symptoms.^{1,2,4,5} Furthermore, excessive weight gain and obesity are associated with increased morbidity from coronary heart disease, diabetes, hypertension, gallbladder disease, and some forms of cancer.⁶ Monitoring and combating antipsychotic drug-induced weight gain may therefore play a part in promoting treatment compliance and general health among psychotic patients.

Psychotropic drugs that influence serotonin (5-HT) neurotransmission have been reported to affect food intake and cause fluctuations in weight. Drugs that facilitate serotonin transmission have been found to reduce food consumption and cause weight loss.⁷⁻⁹ In contrast, drugs that block serotonin transmission have been found to increase food intake and cause weight gain.^{2,10-12}

It remains speculative which serotonin receptor type is responsible for stimulating food intake and weight gain. Aulakh et al.¹³ suggested that both 5-HT_{1C} and 5-HT₂ receptors play an important role in the stimulation of food intake; these receptors are now referred to as 5-HT_{2C} and 5-HT_{2A}, respectively.¹⁴ Tecott and colleagues¹⁵ developed a strain of mice whose gene for the 5-HT_{2C} receptor was removed. These mice became obese and had a propensity for seizures.

In addition to animal models for the role of 5-HT_{2C} in eating behavior, the medication fenfluramine is thought to

Table 1. Binding Affinity In Vitro of Antipsychotics for Neurotransmitter Receptor Subtypes^a

Drug	5-HT _{2A}	5-HT _{2C}	D _{2L}	D _{2S}	α_1	H ₁
Clozapine	9.6	13	192	147	23	0.23
Olanzapine	2.5	7.1	31	21	60	0.65
Risperidone	0.52	48	5.9	6.2	2.3	20
Haloperidol	196	> 10,000	2.2	1.8	19	790
Sertindole	0.39	1.9	7.0	5.8	1.8	130

^aReprinted with permission from reference 14. Binding affinity (K_i) values are shown in nmol/L.

exert its chemical activity as a 5-HT_{2C} (formerly 5-HT_{1C}) agonist, thus suppressing appetite.¹⁶ Additionally, *m*-chlorophenylpiperazine (*m*-CPP), a serotonin agonist, decreases food intake when given to humans.¹⁷ This adds further evidence that the serotonin blockade of novel antipsychotics may do the opposite, i.e., increase food intake.

Also, some of these novel agents, particularly clozapine, have strong affinity for histamine H₁ receptor sites.^{18,19} Links between weight gain, use of antihistamines, and older conventional antipsychotic agents such as thioridazine and chlorpromazine have been demonstrated in humans.^{2,3,20-22}

Clozapine, risperidone, sertindole, and olanzapine are novel antipsychotics that have been developed in an effort to increase antipsychotic efficacy with fewer side effects, particularly extrapyramidal side effects, than conventional antipsychotics. In comparison with conventional antipsychotics, these novel antipsychotics are pharmacologically characterized as potent serotonin receptor antagonists with a lower affinity for dopamine D₂ receptor sites.²³⁻²⁶ Specifically, clozapine, risperidone, sertindole, and olanzapine have high binding affinities for 5-HT_{2C} and 5-HT_{2A}.^{14,27,28} Clozapine also has a large amount of activity at cholinergic and histaminergic receptor sites.^{18,19} Previous studies have indicated that clozapine, risperidone, and olanzapine are responsible for drug-induced weight gain in psychotic patients.²⁹⁻³³ Table 1 shows the medications' binding affinities for neurotransmitter receptor subtypes.

In contrast, haloperidol is a conventional antipsychotic with a low affinity for serotonin receptor binding sites.³⁴ Studies indicate that haloperidol's serotonergic properties are significantly weaker than those exhibited by clozapine, risperidone, sertindole, and olanzapine.^{25,27,35-37} It has been hypothesized that haloperidol's limited serotonergic properties are responsible for the drug's lower potential to induce weight gain² and that the different serotonergic affinities exhibited by the novel antipsychotics and haloperidol might explain the variation in the amount of weight gained by patients receiving these drugs.³⁸

This study is a retrospective analysis of the relative weight gain liabilities of clozapine, risperidone, olanzapine, and sertindole compared with that of haloperidol. We hypothesized that the unique pharmacodynamic profiles of these agents will contribute to different amounts and different patterns of weight gain.

METHOD

Subjects and Procedures

The subjects were 92 male patients with schizophrenia (DSM-III-R criteria) who were participants in 8 different clinical drug trials conducted over 6 years in our research clinic: a study comparing clozapine with haloperidol, 2 different studies comparing risperidone with haloperidol, a study comparing sertindole with placebo, a study comparing olanzapine with placebo, a study comparing 4 dose levels of haloperidol decanoate, a study comparing clozapine with risperidone, and a study comparing olanzapine with risperidone. Thirty subjects participated in more than 1 study over the 6-year period. Thus, 122 clinical records were included in the analyses.

As noted above, some of these clinical trials included placebo controls during the initial double-blind phase. Placebo-control subjects were not entered into these analyses of weight gain because none of them were maintained on treatment with placebo longer than 6 weeks (see Table 2 for average duration of treatment). All studies were double-blind comparisons that were followed by open-label extension phases, with the exception of the haloperidol decanoate study (it had no open-label extension). The patients in the 2 clozapine-treatment studies and 1 of the risperidone versus haloperidol studies were classified as treatment resistant; the patients in 1 of the risperidone studies, the sertindole study, and both olanzapine studies were classified as treatment responsive; and the patients in the haloperidol decanoate study had a history of psychotic decompensation with a need to be maintained on treatment with antipsychotics. The drug studies were all conducted at the V.A. Greater Los Angeles Healthcare System. All the patients provided informed consent after receiving a full explanation of their respective study procedures.

At the time these data were collected, our clinic was running approximately 10 studies. The 8 studies chosen were selected because they had the greatest number of patients enrolled for the longest period of time. These studies have been conducted over a 6-year period.

Medical charts were reviewed to obtain the following information: age, ethnicity, diagnosis, weight changes, duration of treatment, and inpatient status. Demographic variables are summarized in Table 2. Patients were weighed as part of routine procedures using the same clinic scale.

All patients were subject to the following clinical management of weight control during the 6-year period: First, patients were instructed to weigh themselves and report their weight to our research clinical nurse specialist at each visit (every 1 to 4 weeks). If this simple feedback behavioral paradigm failed to maintain their weight (a gain of 10 lb [4.5 kg] in our subjects is generally considered sufficient to warrant further intervention), they were instructed

Table 2. Demographic Variables

Variable	Drug					Pairwise Comparisons
	Clozapine (N = 20)	Olanzapine (N = 13)	Risperidone (N = 38)	Haloperidol (N = 43)	Sertindole (N = 8)	
Age, y, mean \pm SEM**	43.1 \pm 1.0	44.5 \pm 1.2	43.9 \pm 1.0	41.1 \pm 1.0	42.4 \pm 1.4	Clozapine > haloperidol,** risperidone > haloperidol,** olanzapine > haloperidol**
Ethnicity, N (%)						
White	11 (55)	5 (38)	25 (66)	16 (37)	5 (62)	
African American	5 (25)	5 (38)	9 (24)	10 (23)	2 (25)	
Hispanic	4 (20)	1 (8)	1 (3)	11 (26)	0 (0)	
Other	0 (0)	2 (15)	3 (8)	6 (14)	1 (12)	
Treatment duration, wk, mean \pm SEM**	27.2 \pm 8.0	73.1 \pm 9.9	25.8 \pm 5.8	24.7 \pm 5.4	42.5 \pm 12.6	Olanzapine > clozapine,** olanzapine > risperidone,** olanzapine > haloperidol**

** $p \leq .01$.

to keep a detailed diary of all food intake over a several week period. If this failed to maintain or decrease weight, they were then referred to our clinical nutritionist. Subsequent to this, they were referred to the "Wellness Clinic" at our medical center, which involves a more rigorous evaluation of both dietary and exercise habits and adds education, exercise classes, and group support. While it is true that not all subjects availed themselves of these services, there is no reason to think that any one drug group would have more or fewer "uncooperative" patients.

Statistical Methods

Maximal weight gain was defined as the maximum weight a subject obtained at any point in the study minus his initial weight. Percentage weight gain was defined as maximum weight minus initial weight, divided by initial weight. Of note, 12 patients lost weight during the study. Thus, the maximum weight change for these patients was negative. Final weight change was defined as the final weight observed minus the initial weight. Both maximal weight change and final weight change were studied to see if interventions resulted in a change between maximal and final weights. Repeated-measures analyses of variance (ANOVAs) were performed using the SAS statistical package.³⁹

RESULTS

Subjects

There were statistically significant differences between the drug treatment groups on the demographic variables of age ($F = 4.96$, $df = 4,26$; $p = .004$) and duration of treatment ($F = 5.27$, $df = 4,26$; $p = .003$; see Table 2). There were no other statistically significant differences between groups on demographic variables.

Maximal Weight

Controlling for age, treatment duration, and initial weight, we determined average adjusted maximal weights for each group (Table 3). Clozapine- and olanzapine-

treated patients had the highest maximal weight gains compared with other groups ($F = 4.26$, $df = 4,23$; $p = .01$). Figure 1 represents the percentage of subjects on each drug treatment who gained 10% or more of their baseline weight, less than 10%, and had no weight change. Patients taking olanzapine or clozapine gained weight over greater periods of time compared with risperidone- and sertindole-treated subjects ($F = 2.95$, $df = 4,25$; $p = .04$; see Table 3).

Final Weight

Controlling for age, treatment duration, and initial weight, we found that final weight change was different among groups ($F = 5.69$, $df = 4,23$; $p = .003$; Table 4). At the time of final weight measurement, clozapine-associated weight gain remained statistically significantly higher than that for all other medications. No other pairwise differences were seen.

Relationship Between Initial Weight and Weight Gain

Overall, there was no relationship between body weight status at baseline and either the maximum weight gained or the weight at endpoint. Of the 92 subjects, however, 2 who were thin at baseline were clearly outliers. These 2 patients gained 45 lb and 55 lb (20 kg and 25 kg), respectively, during treatment (with baseline weights of 128 lb and 151 lb [58 kg and 68 kg]). Including these outliers in a Pearson product moment correlation calculation gave only a meager suggestion of an inverse correlation between body weight index at baseline and weight gained ($r = -.014$, $p = .19$). Excluding these outliers, however, caused even this suggestion to disappear completely.

Relationship Between Maximal Weight and Final Weight

The correlation between maximal and final weights was high for all drugs (overall: $r = 0.88$, $p = .0001$; clozapine: $r = 0.98$, $p = .0001$; haloperidol: $r = 0.90$, $p = .0001$; sertindole: $r = 0.94$, $p = .0004$) except olanzapine ($r = 0.83$, $p = .0001$) and risperidone ($r = 0.83$, $p = .0001$).

Table 3. Maximum Actual and Adjusted Weight Gain

Variable	Drug					Pairwise Comparisons
	Clozapine	Olanzapine	Risperidone	Haloperidol	Sertindole	
Beginning weight, mean \pm SD						n/a
lb	184.4 \pm 33.5	190.0 \pm 43.6	185.6 \pm 38.0	186.0 \pm 36.2	188.8 \pm 30.1	
kg	83.0 \pm 15.1	85.5 \pm 19.6	83.5 \pm 17.1	83.7 \pm 16.3	84.9 \pm 13.5	
Adjusted time to maximum weight gain, mean \pm SEM, wk**	24.9 \pm 3.1	21.2 \pm 4.1	15.0 \pm 2.3	18.5 \pm 2.1	8.3 \pm 4.9	Clozapine > risperidone,** clozapine > sertindole,** olanzapine > sertindole* n/a
Maximum weight gain, mean \pm SD						
lb	16.8 \pm 13.3	17.8 \pm 13.3	9.1 \pm 7.6	7.7 \pm 9.0	5.6 \pm 7.3	
kg	7.5 \pm 6.0	8.0 \pm 6.0	4.1 \pm 3.4	3.5 \pm 4.1	2.5 \pm 3.3	
Maximum adjusted weight gain, mean \pm SEM**						Clozapine > sertindole,* clozapine > haloperidol,** olanzapine > sertindole,* olanzapine > haloperidol**
lb	15.2 \pm 1.8	15.0 \pm 2.2	11.1 \pm 1.4	8.2 \pm 1.3	6.8 \pm 2.8	
kg	6.9 \pm 0.8	6.8 \pm 1.0	5.0 \pm 0.6	3.7 \pm 0.6	3.1 \pm 1.2	
Maximum % weight gain, mean \pm SD	9.5 \pm 8.1	10.5 \pm 10.3	5.2 \pm 4.4	4.1 \pm 4.7	3.2 \pm 4.5	n/a
Maximum adjusted % weight gain, mean \pm SEM**	8.8 \pm 1.1	8.8 \pm 1.3	6.4 \pm 0.8	4.4 \pm 0.8	4.1 \pm 1.7	Clozapine > sertindole,* clozapine > haloperidol,** olanzapine > sertindole,* olanzapine > haloperidol**

* $p \leq .05$. ** $p \leq .01$.

Relationship Between 5-HT_{2C} Receptor Affinity, H₁ Receptor Affinity, and Weight Gain

No relationship could be established between the relative 5-HT_{2C} receptor affinities and weight gain. However, an exponential relationship was seen between the medications' H₁ receptor affinities and maximum weight gain (Figure 2).

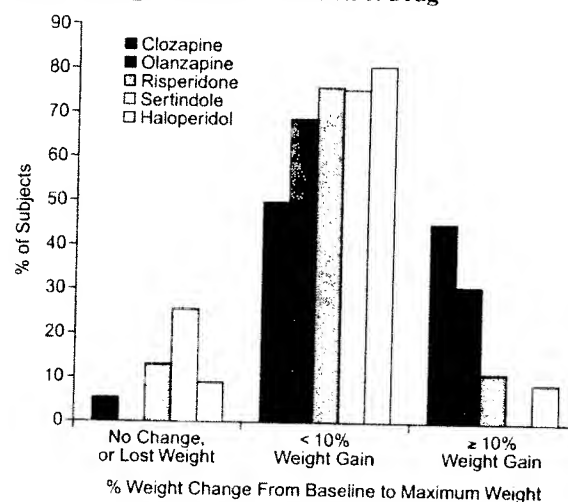
DISCUSSION

Novel antipsychotics vary in their weight gain liabilities. Clozapine and olanzapine appear to cause the most weight gain, risperidone is intermediate, and sertindole actually has less associated weight gain than haloperidol. Clozapine's effect on weight gain was sustained and unresponsive to interventions, whereas olanzapine's weight gain effect was somewhat reversible with dietary and other behavioral maneuvers. However, the possibility that this phenomenon may simply be an artifact of the much longer treatment duration of the olanzapine patients (73.14 weeks) compared with that of the clozapine patients (27.17 weeks) cannot be ruled out.

For those patients who did gain weight, the time course was distinct among treatment groups. Risperidone- and sertindole-treated subjects reached a weight plateau after a comparatively short initial time period (circa 10 weeks), whereas olanzapine- and clozapine-treated patients continued to gain weight over a more lengthy period (circa 20 weeks.)

The weight gain liabilities of these drugs appeared to be correlated with their relative affinities for the histamine H₁ receptor. Despite our speculation that weight gain from these new agents would be linked to serotonin receptor activity, no relationship could be es-

Figure 1. Weight Gain as a Function of Drug



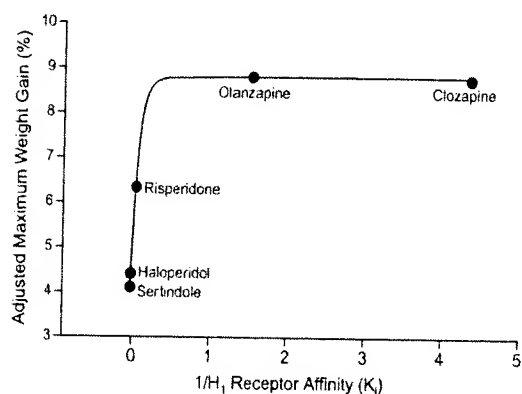
tablished between clinical weight gain and 5-HT_{2C} receptor affinity.

The literature is variable in terms of establishing a link between weight gain and dose of conventional antipsychotic medications.¹⁰⁻⁴³ Our sample size is too small to adequately address the issue of dosage. In future studies, we will attempt to have a larger sample to examine dosage issues.

Other possible explanations for increased weight gain seen in the clozapine-treated patients compared with the haloperidol-treated patients may be that, clinically, the clozapine-treated patients were ill longer and were thus more sedentary. However, over half of the haloperidol-treated subjects in this retrospective study were matched for severity of illness with the clozapine-treated patients.

Table 4. Final Weight Gain

Variable	Drug					Pairwise Comparisons
	Clozapine	Olanzapine	Risperidone	Haloperidol	Sertindole	
Final weight gain, mean \pm SD						n/a
lb	14.1 \pm 13.5	6.2 \pm 14.1	4.2 \pm 9.2	3.0 \pm 10.9	0.5 \pm 8.6	
kg	6.3 \pm 6.1	2.8 \pm 6.4	1.9 \pm 4.2	1.4 \pm 4.9	0.2 \pm 3.9	
Final adjusted weight gain, mean \pm SEM**						Clozapine > risperidone,** clozapine > sertindole,** clozapine > haloperidol,** clozapine > olanzapine*
lb	15.0 \pm 2.3	5.3 \pm 2.9	5.0 \pm 1.8	3.4 \pm 1.7	1.4 \pm 3.7	
kg	6.8 \pm 1.0	2.4 \pm 1.3	2.3 \pm 0.8	1.5 \pm 0.8	0.6 \pm 1.7	
Final % weight gain, mean \pm SD						n/a
Final adjusted % weight gain, mean \pm SEM**	8.0 \pm 8.2	4.2 \pm 9.8	2.5 \pm 5.3	1.6 \pm 5.7	0.6 \pm 5.2	Clozapine > risperidone,** clozapine > sertindole,** clozapine > haloperidol,** clozapine > olanzapine**
	8.7 \pm 1.3	3.3 \pm 1.6	3.1 \pm 1.0	1.9 \pm 1.0	1.2 \pm 2.0	

* $p \leq .05$. ** $p \leq .01$.Figure 2. Weight Gain as a Function of H_1 Affinity^a

^a $y = 4.7[1 - e^{-12.5x}] + 4.1$ where y = adjusted maximum weight gain (%) and x = $1/H_1$ receptor affinity (K_1).

Additionally, the clozapine-treated patients had a better clinical response than the haloperidol-treated subjects.³⁸ Some researchers have postulated that an increase in weight during clozapine treatment is linked with good clinical response.⁴⁴ Although we have clinical response data for all these patient groups, different rating instruments were used to measure response in each study. Each subset of patients was selected for these studies for their different historical patterns of response; thus, comparisons across these studies would be unfair.

As adults age, weight can naturally increase.⁴⁵ We believe the weight gain seen in our patients treated with the new agents exceeds what would be expected as a normal aspect of aging. Indeed, some patients in each group gained over 30 lb (14 kg).

The fact that only men were included in this study is a limitation, as women may have different weight gain patterns. Another potential difficulty in interpreting our results is that 30 patients were involved in more than 1 study. Sequential participation may artificially minimize

the actual weight gain a patient may have experienced with the second medication he was exposed to in the second trial.

A suggestion coming from pharmaceutical companies is that weight gain may be most significant in patients who were initially underweight. However, we examined this in our data set, and we saw no correlation between body weight index at baseline and weight gain.

Future prospective studies could be designed to actually measure caloric intake and assess the types of foods that patients eat, e.g., are they preferentially increasing carbohydrate intake or fat intake?

Although novel antipsychotic drugs have superiority over haloperidol both in increased effectiveness and in reduced side effects,^{46,47} they carry the liability of potential weight gain. Of note, conventional antipsychotic medications were also notorious for this effect, particularly lower-potency agents.^{1,2} Histamine receptor blockade was speculated to play a role in this in the past.⁴⁸ Antihistamines are well known to cause weight gain,^{2,3,20-22} so antihistamine properties of clozapine and olanzapine may, in part, account for the increase in weight caused by these medications.³⁰ Indeed, we saw the strongest correlation between weight gain and relative histamine H_1 receptor affinities of the novel agents.

Clinicians should be aware of this potential liability of the new agents. Patients should have nutritional counseling and referral to exercise programs while taking these medications. Weight should be monitored carefully over the course of treatment. Primary care practitioners, family members, and other caregivers should be alerted to this risk, as the potential complications of weight gain in patients with schizophrenia can be serious. Of note, several of our patients who have had enormous weight gain on treatment with novel antipsychotic medications have developed diabetes.⁴⁹ Greater risk for heart disease may also result in patients who develop significant weight gain. It is essential that we educate our patients to minimize the risks of this important side effect.

Drug names: chlorpromazine (Thorazine and others), clozapine (Clozaril), haloperidol (Haldol and others), olanzapine (Zyprexa), risperidone (Risperdal), thioridazine (Mellaril and others).

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EXHIBIT

13

Extrapyramidal Side Effects, Tardive Dyskinesia, and the Concept of Atypicality

William M. Glazer, M.D.

The most frequent problems associated with the older generation of antipsychotic agents are extrapyramidal side effects (EPS) and tardive dyskinesia. Neuroleptic-induced EPS are thought to be caused by blockade of nigrostriatal dopamine tracts resulting in a relative increase in cholinergic activity; tardive dyskinesia is less well understood but is thought to be a supersensitivity response to chronic dopamine blockade. The leading hypothesis for the mechanism of action of the newer generation of atypical antipsychotics is the presence of a high serotonin-to-dopamine receptor blockade ratio in the brain. When serotonergic activity is blocked—as is the case with atypical antipsychotics—dopamine release increases and balances out the dopamine blockade effect at postsynaptic receptor sites, which results in few or no EPS. Prospective data indicate that the risk of tardive dyskinesia in patients taking atypical antipsychotics is less than that for those taking typical antipsychotics. This article reviews the mechanisms of neuroleptic-induced EPS and tardive dyskinesia and discusses the relationship between these movement disorders and atypical antipsychotic agents.

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The most frequent problems associated with the older generation of antipsychotic agents are extrapyramidal side effects (EPS) and tardive dyskinesia, and few remedies have been found for these neuroleptic-induced movement disorders. The Nithsdale schizophrenia survey,¹ which followed 271 individuals with schizophrenia over a 10-year period, reported a point prevalence of 27% for parkinsonism, 29% for tardive dyskinesia, and 23% for akathisia or pseudoakathisia; only 44% of the patients surveyed had no movement disorder. In the Yale Tardive Dyskinesia Study,² my colleagues and I estimated the risk of persistent tardive dyskinesia in a prospective cohort of 362 chronic psychiatric outpatients who were free of tardive dyskinesia at baseline and were followed for 5 years while taking typical neuroleptic medications. On the basis of the 5-year follow-up using retrospective medication histories, we were able to estimate the cumulative incidence of tardive dyskinesia for up to 25 years of neuroleptic exposure. The years of neuroleptic exposure and estimated risks for tardive dyskinesia were: 0 to 5 years, 31.8%; 5 to 10 years, 49.4%; 10 to 15 years, 56.7%; 15 to 20 years, 64.7%; and 20 to 25 years, 68.4% as shown in

Table 1. These findings suggest that tardive dyskinesia is a public health problem and a source of great concern to the psychiatric community as well.

It should come as no surprise that EPS and tardive dyskinesia are prevalent in patients taking traditional antipsychotics when one considers the manner in which these agents and their effects have been identified. In the past, researchers commonly used behavioral measures that involved movements in animals to define compounds with antipsychotic properties. Similarly, clinicians commonly used movement disorders in patients as indicators of antipsychotic effects. The serendipitous discovery of clozapine—which causes few EPS and little tardive dyskinesia—has provided a means to utilize neuroscientific methods to identify the atypical properties of clozapine and hopefully to create new agents that will avoid unwanted adverse effects. This article will review the mechanisms of neuroleptic-induced EPS and tardive dyskinesia and discuss the relationship between these movement disorders and the new generation of atypical antipsychotic agents.

ATYPICAL ANTIPSYCHOTIC AGENTS AND EPS

Neuroleptic-induced EPS are thought to be caused by the blockade of nigrostriatal dopamine tracts resulting in a relative increase in cholinergic activity.³ Drugs that block cholinergic activity (e.g., antiparkinsonian agents) or drugs that increase striatal dopamine function (e.g., various atypical antipsychotics) correct the biochemical imbalance caused by postsynaptic striatal dopamine blockade.

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Positron emission tomography (PET) and selective radioligands have been used to determine dopamine receptor occupancy induced by neuroleptics in the basal ganglia of drug-treated schizophrenic patients. These studies show that dopamine D₂ occupancy in the basal ganglia is 70% to 90% in patients treated with typical antipsychotic agents at clinically effective doses and that EPS occur with 80% or greater occupancy of the D₂ receptors⁴; less than 60% D₂

blockade may be insufficient to induce a satisfactory antipsychotic response.⁵ Monitoring of antiparkinsonian medications administered during neuroleptic treatment of schizophrenic patients is another method for assessing EPS. In a multicenter double-blind placebo-controlled study evaluating the efficacy and safety of sertindole and haloperidol in 497 hospitalized schizophrenic patients, there was a clear dose-response relationship between increasing doses of haloperidol and the use of antiparkinsonian agents.⁶

The pathophysiology of tardive dyskinesia is less well understood but is thought to be a supersensitivity response to chronic dopamine blockade—i.e., the blockade of receptors in the nigrostriatal dopamine pathway causes a proliferation of dopamine receptors on the postsynaptic side of the nigrostriatal tract. It is thought that this state of chronic dopamine supersensitivity is manifested clinically as tardive dyskinesia.⁷ Although the theory is inconclusive⁸ and unsupported by research, it remains the leading explanation for the development of tardive dyskinesia and is heuristically useful.

What is the mechanism of action of atypical antipsychotics? The leading hypothesis is the presence of a high serotonin-to-dopamine receptor blockade ratio in the brain⁵; while the various atypical antipsychotics differ in receptor activities, they all share in common a ratio of serotonin-to-dopamine blockade greater than 1. There is evidence for serotonin/dopamine interactions at the level of the basal ganglia in animal models, and serotonergic blockade is thought to play a role in the reduction of risk for EPS associated with atypical agents.⁹ Under normal conditions, presynaptic blockade at the serotonin receptor site inhibits or curbs dopamine release. When serotonergic activity is blocked—as is the case with atypical antipsychotics—it is thought that dopamine release increases and balances out the dopamine blockade effect at postsynaptic receptor sites, resulting in few or no EPS. Thus, in essence, serotonin blockade brings the system into balance.

Table 1. Estimated Risk of Tardive Dyskinesia (TD) (and 95% Confidence Intervals)^a by Net Years of Previous Neuroleptic Use (Without TD) and Additional Years Taking Neuroleptics: Results of the Yale TD Study, 1985–1990^b

Years of Previous Neuroleptic Use	Additional Years Taking Neuroleptics				
	5	10	15	20	25
0	0.318 (0.225, 0.429)	0.494 (0.396, 0.592)	0.567 (0.468, 0.662)	0.647 (0.546, 0.736)	0.684 (0.579, 0.774)
5	0.258 (0.177, 0.360)	0.366 (0.266, 0.478)	0.482 (0.369, 0.598)	0.537 (0.411, 0.658)	
10	0.145 (0.072, 0.270)	0.302 (0.189, 0.445)	0.376 (0.241, 0.533)		
15	0.184 (0.092, 0.333)	0.270 (0.145, 0.446)			
20	0.106 (0.030, 0.315)				

^aRisk estimates are based on the density method, conditional on the number of net years of previous use; confidence-limit estimates are based on a modification of Rothman's method.

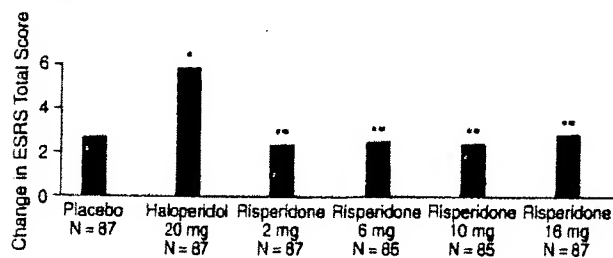
^bFrom reference 2, with permission.

Risperidone and Clozapine

Simpson and Lindenmayer¹⁰ analyzed data from both arms^{11,12} of the North American multicenter comparison of risperidone (2 mg, 6 mg, 10 mg, or 16 mg/day), haloperidol (20 mg/day), or placebo for 8 weeks in 253 chronic schizophrenic patients. The severity of EPS was assessed by the Extrapyramidal Symptom Rating Scale (ESRS), and at the clinically most effective risperidone dose (6 mg/day) the mean ESRS change score was not significantly different from that of the placebo group. A significant linear relationship was noted between change scores and increasing risperidone doses on 4 of the 12 ESRS subscales; nevertheless, even at 16 mg/day, mean change scores were lower than in the haloperidol group (Figure 1). A linear relationship between an increasing risperidone dose and administration of antiparkinsonian medications was also apparent.

In a PET analysis of central dopamine receptor occupancy in patients treated with traditional antipsychotics and clozapine,⁴ a total of 22 schizophrenic patients treated with conventional doses of various classical neuroleptics demonstrated D₂ occupancy of 70% to 80% in the basal ganglia; patients with acute EPS had a higher D₂ occupancy than patients without EPS. In 5 patients treated with clinically effective doses of clozapine, a lower D₂ occupancy of 38% to 63% was observed, and this finding was thought to correlate with the atypicality of the drug and the low frequency of EPS. A PET study of 9 risperidone-treated patients¹³ showed that the mean level of D₂ receptor occupancy per drug dose was 66% at 2 mg/day, 73% at 4 mg/day, and 79% at 6 mg/day. Three patients with the highest receptor occupancies exhibited mild EPS although none required antiparkinsonian medications. The emergence of EPS at higher levels of D₂ receptor occupancy suggests that the high 5-HT₂ affinity of risperidone provides a relative protection only from EPS; once the D₂ occupancy exceeds a certain threshold, that protection may be lost. The implication of these clinical studies and PET data point to a ceiling dose for risperidone, perhaps at 6 mg/day.

Figure 1. ESRS for Placebo, Risperidone, and Haloperidol (change in total score)*



*Data from reference 10. Abbreviation: ESRS = Extrapyramidal Symptom Rating Scale.

*p < .001 vs. placebo.

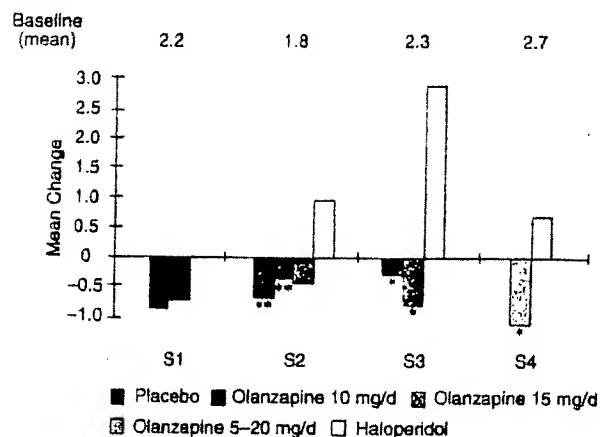
**p < .001 vs. haloperidol.

Olanzapine

Beasley et al.¹⁴ summarized the data on the clinical safety of olanzapine from 4 studies of schizophrenia involving 2500 olanzapine-treated patients, 810 haloperidol-treated patients, and 236 placebo patients. Figure 2 shows the mean change in Simpson-Angus Scale (SAS) scores for acute EPS occurring in patients who participated in the studies. In all 4 trials, there was an improvement over baseline in the SAS analysis for EPS in both mid- and high-dose groups of olanzapine-treated patients compared with haloperidol-treated patients. In study 2,¹⁵ the 5- and 10-mg/day olanzapine doses were significantly less associated with EPS than the 10- and 20-mg/day haloperidol doses. Also in study 2, baseline SAS scores in the 15-mg/day olanzapine-treated patients were not statistically significantly different from those of haloperidol-treated patients, but the patients taking olanzapine showed improvement in EPS while patients taking haloperidol showed worsening of symptoms.

A recent PET study by Kapur et al.⁵ investigated the binding characteristics of olanzapine to 5-HT₂ and D₂ receptors in 15 schizophrenic patients. Imaging of randomly-assigned patients showed that the D₂ striatal occupancy increased with dose; 12 patients taking olanzapine, 5 mg/day to 20 mg/day, showed 43% to 80% D₂ occupancy while 3 patients taking 30 mg/day to 40 mg/day showed 83% to 88% occupancy. The authors concluded that olanzapine is a potent 5-HT₂ blocker and shows a higher 5-HT₂ than D₂ occupancy at all doses; the D₂ occupancy of olanzapine is higher than that of clozapine and similar to that of risperidone. In the usual clinical dose range (10–20 mg/day) of olanzapine, the D₂ occupancy varies from 71% to 80%, which may account for the freedom from EPS in patients who take this agent.

Clinicians should be forewarned that studies involving PET scans may fail to accurately define patients' clinical status because of the following circumstances: (1) medications are usually administered for a few days only prior to scans, (2) there may be interpatient variability of blood lev-

Figure 2. Simpson-Angus Scale Changes in Acute Extrapyramidal Symptoms in 4 Olanzapine Studies³

*From reference 14, with permission. Last observation carried forward.

*p < .001 vs. haloperidol.

**p < .05 vs. haloperidol.

els per drug dose, and (3) studying the striatum may reflect EPS but not efficacy. Therefore, it is difficult to draw firm conclusions about the relationship of dose, blood levels, and clinical response in PET studies. Thus far, the implication of clinical studies and PET data is that the ceiling dose of olanzapine is less clear than risperidone; additional studies of high dose ranges of olanzapine are needed.

Olanzapine Versus Risperidone

Olanzapine and risperidone, both second-generation atypical antipsychotics, differ by virtue of their chemical structure, spectrum of receptor binding affinities, animal neuropharmacology, pharmacokinetics, and in vivo neuroimaging profile. Thus, it was hypothesized that the 2 compounds would show distinct safety and/or efficacy characteristics. To test this hypothesis, an international multicenter double-blind, 28-week prospective study was conducted involving 339 patients who met DSM-IV criteria for schizophrenia, schizophreniform disorder, or schizoaffective disorder.¹⁶ Treatment-emergent EPS—assessed by the SAS and the Barnes Akathisia Scale—were fewer in olanzapine-treated patients (mean dose = 17 mg/day) than in risperidone-treated patients (mean dose = 7 mg/day); these findings also coincided with greater use of anticholinergic medications in risperidone-treated patients. The authors stated that a lower dose range of risperidone and a slower dose titration might have reduced the incidence of EPS in risperidone-treated patients. However, the question remains whether low doses of risperidone will maintain efficacy over time.

Most traditional neuroleptics have a narrow therapeutic-to-toxic index; that is, the separation between the dose that produces efficacy and the one that produces EPS and other

adverse effects is narrow.¹⁷ Researchers who developed the novel antipsychotic drugs set out to substantially widen the distance between the dose that treats psychosis and the one that produces adverse effects.^{18,19} Available data and clinical experience suggest that the therapeutic dose threshold for EPS may be wider for olanzapine than for risperidone. Multiple fixed-dose comparisons of the 2 drugs are needed to verify this preliminary impression.

Quetiapine

Arvanitis et al.²⁰ used 5 fixed doses (75, 150, 300, 600, or 750 mg/day) of the atypical antipsychotic quetiapine to delineate a dose-response relationship and to compare efficacy and tolerability opposite a fixed dose (12 mg/day) of haloperidol and placebo. A total of 361 patients from 26 North American centers with diagnoses of acute exacerbation of chronic schizophrenia per DSM-III-R criteria entered the double-blind, placebo-controlled trial. None of the quetiapine-treated patients—contrasted with 4 haloperidol-treated patients and 1 placebo patient—were withdrawn from the study because of EPS. Moreover, unadjusted mean changes from baseline to endpoint SAS total scores were negative, indicating improvement in EPS for all patient groups except the haloperidol group. Accordingly, the ceiling dose of quetiapine relative to EPS remains unclear.

ATYPICAL ANTIPSYCHOTIC AGENTS AND TARDIVE DYSKINESIA

Do Fewer EPS Predict Less Tardive Dyskinesia?

Clinicians and researchers have long been interested in the relationship between tardive dyskinesia and EPS. As stated above, neuroleptic-induced EPS are likely caused by blockade of nigrostriatal dopamine tracts,³ and tardive dyskinesia is thought to be a supersensitivity response to chronic dopamine blockade.⁷ The supersensitivity theory would predict that patients who develop early neuroleptic-induced EPS will develop tardive dyskinesia. As early as 1972, Crane²¹ hypothesized that tardive dyskinesia is more likely to occur in patients who experience early EPS. The clinical logic underlying this hypothesis was that the appearance of EPS early in the course of exposure to neuroleptic medications was indicative of pathology that would eventually evolve into tardive dyskinesia. Direct support for this hypothesis can be found in various incidence studies.

Kane et al.²² compared the incidence of tardive dyskinesia in 369 relatively young neuroleptic-treated patients who had no history of EPS with that of 52 neuroleptic-treated patients who exhibited severe EPS; patients in the latter group were 2.3 times more likely to develop tardive dyskinesia. The strongest data supporting the hypothesis were reported by Saltz et al.,²³ who investigated the incidence of tardive dyskinesia in elderly individuals just beginning treatment with antipsychotic drugs. Researchers found that the presence of EPS early in treatment was a

predictor of tardive dyskinesia in a newly-exposed elderly population. These findings were replicated by Jeste et al.,²⁴ who studied 266 middle-aged and elderly outpatients with a median duration of 21 days of total lifetime neuroleptic exposure prior to study entry. Patients were treated with either a high- or low-potency neuroleptic and maintained at relatively low doses. The cumulative incidence of tardive dyskinesia was 26%, 52%, and 60% after 1, 2, and 3 years of treatment, respectively.

Two incidence studies found no evidence for support of the hypothesis. Chatterjee et al.²⁵ found no correlation in the prevalence of EPS and spontaneous dyskinesia in neuroleptic-naïve, first-episode schizophrenic patients. However, the follow-up period may have been too short to identify enough incident cases of tardive dyskinesia to demonstrate a relationship between the 2 adverse movement disorders. Among long-term outpatients maintained with neuroleptic medications in the Yale Tardive Dyskinesia Study,²⁶ our group found no baseline relationship of tardive dyskinesia to antiparkinsonian drug use and no clinical findings of EPS or history of EPS. This study may have failed to identify such a relationship because the clinical history was retrospective and may not have accurately identified early EPS.

If an antipsychotic agent causes few EPS—and if few EPS early in treatment are prognostic of less tardive dyskinesia—will the antipsychotic cause less tardive dyskinesia? Prospective data indicate that the risk for tardive dyskinesia is less with administration of the atypical antipsychotic agents clozapine and olanzapine than for the neuroleptic haloperidol.

Clozapine

To determine if chronic exposure to clozapine could cause tardive dyskinesia, Kane et al.²⁷ utilized prospective data on abnormal involuntary movements derived from 28 at-risk schizophrenic or schizoaffective patients who had received clozapine for at least 1 year. The patients were monitored with the modified Simpson Dyskinesia Scale every 3 months, and findings were compared with another group of patients with similar diagnoses who were treated with a variety of typical neuroleptics for at least 1 year. Two patients in the clozapine-treated group—both of whom had ratings of questionable tardive dyskinesia at baseline—were rated as having mild tardive dyskinesia. A survival analysis comparing patients in the clozapine-treated group with those in the neuroleptic-treated group showed a lower risk of tardive dyskinesia developing in the clozapine-treated group; however, the authors were unable to definitely conclude whether chronic exposure to clozapine could cause tardive dyskinesia.

Olanzapine

In a recently-published, long-term, follow-up study, Beasley et al.²⁸ utilized data from a double-blind extension

Table 2. Incidence Rate/Year of Tardive Dyskinesia: Olanzapine Versus Haloperidol^a

Treatment	N	N with TD	Incidence Rate/y
Olanzapine	513	2	0.006
Haloperidol	114	5	0.072

^aAdapted from reference 28. Abbreviation: TD = tardive dyskinesia.

of 3 studies to compare incidence rates of tardive dyskinesia in 513 olanzapine-treated patients and 114 haloperidol-treated patients. Abnormal Involuntary Movement Scale examinations were performed twice weekly to weekly in the 6-week acute phase and every 2 weeks to every 2 months during the extension phase. Cases of tardive dyskinesia that occurred during the first 6 weeks were eliminated from the study, because researchers attributed them to either withdrawal cases or overlooked baseline cases. In doses of 5 mg to 20 mg/day, 2 olanzapine-treated patients—compared with 5 haloperidol-treated patients—developed tardive dyskinesia; thus, haloperidol-treated patients had an estimated tardive dyskinesia incidence rate/year 12 times higher than that of olanzapine-treated patients (Table 2). Patients included in this study had long histories of illness and treatment with antipsychotics, and incidence rates should ultimately be evaluated in prospective comparison studies among drug-naïve patients. In summary, studies of the new antipsychotic medications point to a lower risk for development of tardive dyskinesia. If these studies are accurate, atypical antipsychotic agents will quickly replace the traditional agents.

CONCLUSION

What are the considerations for use of atypical antipsychotics as standard care in the treatment of schizophrenic patients in the present clinical environment? Definition of a patient's *stability* is changing because of evidence that atypical agents improve functioning and promote rehabilitation. Thus, the "stable" patient, i.e. the one who is staying out of the hospital, may now be able to function better. He or she should be able to move toward greater independence and maximal potential. At a minimum, patients should be informed about the option of taking atypical antipsychotics; if the risk of relapse from switching drugs is weighed by the physician and felt to be manageable, treatment with the new antipsychotics should be considered. In unstable or first-break schizophrenics or those who have been untreated for a long period of time, atypical antipsychotics are the drugs of choice.

Extrapyramidal symptoms and tardive dyskinesia are commonly associated with the use of traditional neuroleptics and are a source of great concern to the psychiatric community. The mechanism of neuroleptic-induced EPS is thought to be blockade of nigrostriatal dopamine tracts that results in an increase in cholinergic activity; tardive dyskinesia is thought to be the clinical manifestation of a

supersensitivity response to chronic dopamine blockade. The new generation of atypical antipsychotics shares a common serotonin-to-dopamine blockade ratio that may play a role in reduction of risk of EPS. Early studies are showing a diminished risk of tardive dyskinesia with atypical in comparison to typical antipsychotics. While additional studies are indicated, these results should have a profound impact on clinical practice. Data and clinical studies are emerging that support the first-line use of atypical antipsychotics in patients with schizophrenia. In today's clinical environment, patients should be apprised of the advantages of the new agents and given the option of taking the medications if the attending physician believes the risk of switching drugs is manageable.

Drug names: clozapine (Clozaril and others), haloperidol (Haldol and others), quetiapine (Seroquel), risperidone (Risperdal), olanzapine (Zyprexa).

Disclosure of off-label usage: The author of this article has determined that, to the best of his knowledge, no investigational information about pharmaceutical agents has been presented herein that is outside U.S. Food and Drug Administration-approved labeling.

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EXHIBIT

14

Current Concepts of Pharmacotherapy in Hypertension

Domenic A. Sica, MD, Senior Editor

Alpha₁-Adrenergic Blockers: Current Usage Considerations

Domenic A. Sica, MD

Alpha₁-adrenergic-blocking drugs are effective in reducing blood pressure and do so in a fashion comparable to most other antihypertensive drug classes. These compounds are most effective in patients in the upright position, reducing systolic and diastolic pressures by 8%–10%. Alpha₁-adrenergic-blocking drugs incrementally reduce blood pressure when combined with most drug classes and are the only antihypertensive drug class to improve plasma lipid profiles. Alpha₁-adrenergic-blocking drugs are also accepted as important elements of the treatment plan for symptomatic benign prostatic hypertrophy. Dose escalation of an α_1 -adrenergic-blocking drug can trigger renal Na⁺ retention, and the ensuing volume expansion can attenuate its blood pressure-lowering effect. Orthostatic hypotension can occur with these compounds, particularly when a patient is volume-contracted. Dizziness, headache, and drowsiness are common side effects with α_1 -adrenergic blockers. A modest decline in the use of doxazosin and other α_1 -adrenergic-blocking drugs has occurred coincident to the early termination of the doxazosin treatment arm in the

Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial. (J Clin Hypertens. 2005;7:757–762) ©2005 Le Jacq Ltd.

Selective α_1 -adrenergic blockers retain a small market share among all antihypertensive medications in the United States.^{1,2} Prazosin, released in 1976, was the first marketed drug in this class. Since then two additional α_1 -adrenergic blockers, doxazosin and terazosin, have become available, and their potential for once-daily dosing has provided additional treatment flexibility.³ More recently, sustained-release formulations for prazosin and doxazosin have been released. Tamsulosin and alfuzosin are so-called uroselective agents with a higher affinity for prostatic α_1 adrenoceptors and are commonly used in the management of patients with benign prostatic hypertrophy (BPH).⁴

RECEPTOR SUBTYPES

Alpha adrenoceptors have been divided into α_1 and α_2 receptors. Multiple α_1 and α_2 adrenoceptor subtypes exist. Relevant to this discussion, three α_1 adrenoceptor subtypes have been cloned and are designated α_{1A} , α_{1B} , and α_{1D} . Alpha₁ adrenoceptors are localized postsynaptically in smooth muscle adjacent to nerve terminals. When these α_1 receptors are stimulated by endogenously released norepinephrine, vasoconstriction occurs in both arteries and veins.

After extensive characterization of cloned and native receptors in diverse tissues, it remains difficult to ascribe a definite clinical purpose to each α_1 adrenoceptor subtype beyond the role of α_1 adrenoceptor stimulation in the symptom profile

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NOTICE

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Bonhaus Declaration,

Exhibit 14

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of BPH. There is evidence that tamsulosin and alfuzosin have a higher affinity for α_{1a} and α_{1d} adrenoceptor subtypes. Binding studies have shown that tamsulosin is as much as 12 times more selective for α_1 adrenoceptors in human prostate tissue than for α_1 adrenoceptors in the aorta.⁵ Tamsulosin and alfuzosin are approved as uroselective agents for the treatment of symptomatic BPH.⁶

MECHANISM OF ACTION

Alpha₁-adrenergic-specific antagonists inhibit the vasoconstrictor effect of norepinephrine. They do so by selectively inhibiting the activation of postsynaptic α_1 receptors by circulating and/or neurally released catecholamines. The drop in peripheral vascular resistance that follows is the basis for the ensuing blood pressure (BP) fall. Alpha₁-adrenergic antagonists have little-to-no effect on cardiac output or renin release, in part because of a balanced effect on venous and arteriolar tone.⁷ The presynaptic α_2 -adrenergic receptor goes unblocked with these selective compounds; therefore, inhibition of additional norepinephrine release by a feedback mechanism of α_2 -adrenergic receptor stimulation is preserved.

This inhibition of norepinephrine release explains the absence of tachycardia, increased cardiac output, and rise in renin levels that characterize drugs that block both the presynaptic α_2 receptor and the postsynaptic α_1 receptors (e.g., phentolamine).⁸ Because these agents do not interfere with the renin-angiotensin-aldosterone system, they may be a practical choice for the control of hypertension in patients undergoing dynamic testing of this axis.⁹ These drugs may also have beneficial effects on hemorheology,¹⁰ including blood viscosity, red blood cell deformability, and endothelial function.¹¹ Doxazosin also inhibits the proliferation and migration of human vascular smooth muscle cells, independent of α_1 -adrenoceptor blockade.¹²

Baroreceptor reflexes as well as supine and upright heart rate do not ordinarily change with α_1 -adrenergic antagonist therapy. Orthostatic hypotension can occur in α_1 -adrenergic antagonist-treated patients who are either volume-depleted or hemodynamically sensitive to the loss of α_1 -adrenergic-mediated vasoconstriction that might otherwise occur with assumption of an upright posture. The effectiveness of α_1 -adrenergic antagonists is proportional to the level of sympathetic activation in the treated patient. This is why these drugs will not reduce BP in normotensive persons at a normal level of sympathetic nervous system activity.

AVAILABLE AGENTS

Both selective and nonselective α_1 -adrenergic antagonists are clinically available. Phenoxybenzamine is a noncompetitive, nonselective α blocker that is now reserved for the preoperative management of pheochromocytoma-related hypertension. Nonselective α blockade means that presynaptic α_2 receptors, which reduce the release of norepinephrine, are inhibited because the negative feedback mechanism is blocked. Phentolamine is a short-acting, competitive, nonselective α blocker parenterally administered and used almost exclusively for urgent, severe forms of hypertension prompted by excessive catecholamine release.

Prazosin was the first selective α blocker. This compound has a high affinity for the α_1 receptor, and when given as an immediate-release formulation it has a rapid onset of action. This feature probably accounts for its relatively higher rate of syncope and orthostatic hypotension compared with doxazosin and terazosin. Syncopal episodes can be minimized by limiting the initial dose to 1 mg, administering the first dose at bedtime, and increasing the dosage slowly.¹³ Both terazosin and doxazosin are less lipid-soluble than prazosin and have a lower affinity for α_1 receptors.

The individual members of the α_1 -adrenergic antagonist class are pharmacologically distinct (Table I).^{14–16} Prazosin has a relatively short duration of action and should be given at least twice daily.¹⁴ Terazosin and doxazosin have longer half-lives and can be administered once daily.^{15,16} Doxazosin can be administered at bedtime, with its pattern of slow absorption allowing for a maximal effect on the early morning surge in BP.¹⁷ In general, α_1 -adrenergic antagonists should be used cautiously in children or in pregnancy, since the efficacy and/or safety of these compounds have not been evaluated in these patient types (Table II).

BP-LOWERING EFFICACY

The degree to which BP is reduced with α_1 -adrenergic antagonists is comparable to that observed with other major classes of antihypertensive medications including diuretics, β blockers, angiotensin-converting enzyme (ACE) inhibitors, and calcium channel blockers (CCBs).^{18–25} Alpha₁-adrenergic antagonists reduce systolic and diastolic BP by approximately 10% and are more effective in reducing upright than supine BP. Most BP lowering occurs at low- to mid-range doses, such as 4–8 mg/d of doxazosin and 5–10 mg/d of terazosin.²⁶ Higher doses bring about Na⁺ retention, perhaps because plasma renin activity and plasma

Table I. Pharmacokinetics of Selective α_1 -Adrenergic Antagonists

DRUG	DAILY DOSE (MG)	DOSES PER DAY	BIOAVAILABILITY (%)	HALF-LIFE (H)	URINARY EXCRETION (%)
Prazosin	2–20	2–3	44–69	2.5–4	10
Terazosin	1–20	1	90	12	10
Doxazosin	1–16	1	65	19–22	5

aldosterone do not suppress as completely with α_1 -adrenergic antagonists as they do with other adrenergic-inhibiting drugs.²⁷

Alpha₁-adrenergic antagonists are most effective in low and medium plasma renin activity states.²⁸ Studies have been inconsistent as to the effect of age and race on the BP-lowering response to these drugs.^{18,19,29} Alpha₁-adrenergic antagonists may find their greatest use as add-on therapy to other antihypertensives. These compounds reduce BP significantly when added to multiple antihypertensive medication classes, oftentimes controlling BP in patients resistant to two or more therapies.^{30,31}

ALPHA₁-ADRENERGIC ANTAGONISTS AND TREATMENT GUIDELINES

For many years α_1 -adrenergic antagonists had been considered suitable initial drugs for uncomplicated early-stage hypertension. More recently, guideline-generating groups including the European Society of Hypertension/European Society of Cardiology and the authors of the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) no longer include α_1 -adrenergic antagonists as initial agents.^{32,33} This removal of α_1 -adrenergic antagonists from initial therapy status related to findings from the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT).^{34,35}

ALLHAT was a large, simple trial that studied high-risk hypertensive patients aged 55 years or older. Its goals were to determine whether the incidence of the *primary outcome*—a composite of fatal coronary heart disease and nonfatal myocardial infarction—differed between treatment with a diuretic (chlorthalidone, 12.5–25.0 mg/d) and treatment with each of three other types of antihypertensive drugs: a CCB (amlodipine), an ACE inhibitor (lisinopril), and an α -adrenergic blocker (doxazosin, 2–8 mg/d). In this study, doxazosin titration occurred on a monthly basis. *Secondary outcomes* included all-cause mortality, stroke, and all major cardiovascular (CV) disease events. If patients did not meet the BP goal with the maximum tolerated dose of the initial medication, an open-label step 2 agent (atenolol, 25–100 mg/d; reserpine, 0.05–0.2 mg/d; or clonidine, 0.1–0.3 mg

Table II. Pharmacokinetics of Selective α_1 -Adrenergic Antagonists in Special Populations

DRUG	PREGNANCY CATEGORY	BREAST MILK TRANSMISSION	PEDIATRIC STUDIES
Prazosin	C	Yes	No
Terazosin	C	Not known	No
Doxazosin	C	Yes	No

C=risk cannot be ruled out; adequate studies lacking

b.i.d.) or an open-label step 3 agent (hydralazine, 25–100 mg b.i.d.) could be added.³⁴

In ALLHAT there was no difference in the primary outcome of fatal/nonfatal myocardial infarction or all-cause mortality when the doxazosin-based regimen was compared with one utilizing chlorthalidone. The doxazosin treatment arm of this study was terminated early, however, because increased CV end points were seen when compared with chlorthalidone. There was a 19% excess stroke incidence with doxazosin and a highly significant increase (25%) in combined CV disease. There was also a 66% increase in fatal or hospitalized heart failure in the doxazosin group, which was a major contributor to the increase in combined CV disease.^{34,35} The basis for the increased incidence of doxazosin-related heart failure in ALLHAT remains uncertain despite a number of explanations having been proffered. Etiologic considerations in this process include plasma volume expansion, sympathetic nervous system activation, inadequate BP control, and suboptimal regression of left ventricular mass.^{36,37} What remains to be more carefully elucidated is the exact interplay of these variables in the final outcome of ALLHAT.

ALPHA₁-ADRENERGIC ANTAGONISTS AND PRESCRIPTION TRENDS

As a consequence of the ALLHAT findings, the number of prescriptions for α_1 -adrenergic antagonists has fallen off significantly (Figure).^{1,2} Between 1999 and 2002, new annual α -blocker prescription orders declined by 26%, from 5.15 to 3.79 million, dispensed prescriptions dropped by 22% from 17.2 to 13.4 million, and physician-reported drug use fell by 54%, going from 2.26 to 1.03 million. This downward trend in α_1 -adrenergic antagonist use is further supported by a recent audit of a

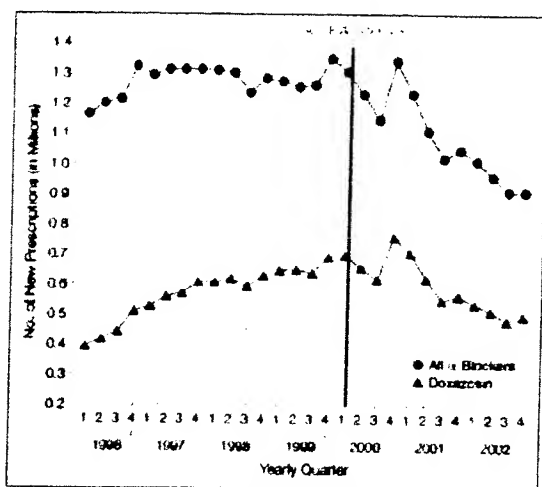


Figure. Trends in new α blocker and doxazosin prescriptions received by retail pharmacies. Data are from the National Prescription Audit Plus from IMS Health (Plymouth Meeting, PA) and are based on new prescriptions from January 1996 through December 2002. "ALLHAT Results" indicates the release date of findings from the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial. Reproduced with permission from JAMA. 2004;291:54-62.¹

large computer-stored prescription information base from the Kaiser Permanente of Southern California health maintenance organization.²

SIDE EFFECTS

In long-term, well controlled studies, the side-effect rates with α_1 -adrenergic antagonists compare favorably with those observed with other major drug classes.^{38,39} For most patients, the side effects of α_1 -adrenergic antagonists are highly dose-dependent but tend to diminish with continued therapy. The most distressing side effect with α_1 -adrenergic antagonists has been first-dose hypotension or syncope, which is seen most frequently with shorter-acting agents, in volume-depleted states, and with higher doses of these compounds.³⁹ For example, in the Treatment of Mild Hypertension Study (TOMHS),²⁰ the reported incidence of syncope was no different than that observed with placebo; however, only a 2-mg dose of doxazosin was given. Alternatively, giving more than 4 mg of doxazosin significantly increases the likelihood of postural dizziness/vertigo, hypotension, and syncope. Alpha₁-adrenergic antagonists do not routinely cause impotence. In TOMHS, the incidence of erectile dysfunction with doxazosin was similar to that with placebo.¹⁸ In women, urinary incontinence may be triggered by α_1 -adrenergic antagonists, a side effect that is reversible on withdrawal of the offending drug.⁴⁰

METABOLIC AND ANCILLARY EFFECTS OF α_1 -ADRENERGIC ANTAGONISTS

Alpha₁-adrenergic antagonists are the only class of antihypertensive agents that have consistently been shown to favorably affect plasma lipids^{23,41-43} and insulin sensitivity.^{23,43-45} Total cholesterol and low-density lipoprotein cholesterol are both lowered by approximately 5%, triglycerides by 10%, and in some studies high-density lipoprotein cholesterol increases with drugs in this class.⁴¹⁻⁴³ An example of this beneficial metabolic effect can be found in the study by Andersson and Lithell²³ of 43 hypertensive and hypertriglyceridemic patients treated with either doxazosin or enalapril. Over a 6-month period, both agents provided similar BP reduction as determined by 24-hour ambulatory BP monitoring. Doxazosin, more so than enalapril, however, significantly reduced serum lipids, and increased lipoprotein lipase activity and the elimination rate of an IV-administered fat load, while improving insulin sensitivity. Doxazosin has also been shown to improve fibrinolysis. Its administration is accompanied by a lowering of plasminogen activator inhibitor-1 activity and higher tissue plasminogen activator activity after venous occlusion.⁴⁶

Alpha₁-adrenergic antagonists do not adversely affect renal function²⁵ and, like a number of other agents, they regress left ventricular hypertrophy,^{24,47-49} although regression of left ventricular hypertrophy with α_1 -adrenergic antagonists may be less than that seen with hydrochlorothiazide, captopril, or atenolol.⁵⁰ During isotonic exercise, these drugs will not reduce the exercise-associated rise in systolic BP as well as β blockers⁵¹; alternatively, α_1 -adrenergic antagonists will suppress the pressor response better during isometric exercise than will a β blocker.⁵²

Alpha₁-adrenergic antagonists have emerged as an effective form of therapy for symptomatic BPH.⁴⁻⁶ Alpha-adrenergic receptors have been identified in the bladder neck and prostatic capsule, and their stimulation is responsible for the dynamic pressure component of BPH symptoms. In clinical studies, use of α_1 -adrenergic blockers in patients with BPH increases urinary flow rate and reduces residual volume and obstructive symptoms.^{4,6} In those BPH patients who are hypertensive, BP will fall in a dose-dependent fashion with α_1 -adrenergic antagonists; however, single-drug therapy with an α_1 -adrenergic antagonist—for BP control and BPH symptom relief—may require doses larger than those used for the treatment of patients with BPH alone. The most recent American Urologic Association guidelines on the management of BPH

advocate the use of an α_1 blocker to manage a patient's lower urinary tract symptoms; however, such therapy should not be presumed per se to represent a best-care strategy for management of a patient's hypertension, if also present.⁵³

FUTURE TRENDS WITH α_1 -ADRENERGIC ANTAGONISTS

Alpha-adrenergic blocking drugs have been used with considerable success in the treatment of hypertension over the past two decades. Much of their past success has occurred when they have been used in resistant hypertensives as add-on therapy to drug classes such as ACE inhibitors or CCBs. The future for this drug class will not change in that regard, particularly since overactivity of the sympathetic nervous system, either on a primary or secondary basis, is commonplace in resistant forms of hypertension.

Another area of use for α_1 -adrenergic blocking drugs will be in the treatment of the male hypertensive with BPH symptoms. These compounds typically improve both symptom score and urinary flow in men with BPH. Uroselective α -adrenergic-blocking drugs have been developed to treat individuals with BPH and, for the most part, they do not adversely affect BP. Uroselective α -adrenergic-blocking drugs are important treatment tools for the hypertensive patient with BPH, since such patients are best treated by the separate management of each condition—as has been suggested by the American Urologic Association and JNC 7.⁵³ This highly selective form of BPH therapy typically sidesteps the vasodepressor risk of α_1 -adrenergic-blocking drugs in normotensive patients.

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EXHIBIT

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Exhibit 15

Receptor Selection and Amplification Assay (RSAT™) Protocol to Assess Off-Target Inverse Agonist and Antagonist Activities

Inverse Agonist Activity in Human 5-HT_{2B} Receptors

Receptor Selection and Amplification (RSAT™) functional assays were carried out essentially as described in *Weiner et al., J. Pharmacol. & Exp. Therap.*, Vol. 299, No.1, pp. 268-79 (2001) (included herewith as **Exhibit 9**) with some modification. NIH-3T3 cells were grown to 70-80% confluence in large vessels in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum and 1% penicillin/ streptomycin/L-glutamine (PSG). Cells were transfected with plasmid encoding human 5-HT_{2B} receptor DNA, DNA for G_q G-protein subunit and DNA for beta-galactosidase gene using superfect reagent. After 18-20 hours, the cells were harvested, aliquoted and frozen at -80°C until use. On the day of the assay, the cells were thawed and plated onto 96 well plates containing varying concentration of the ligand under investigation. Cells were incubated in a humidified atmosphere with 5% ambient CO₂ for 5 days. Media were removed from the plates, and beta-galactosidase activity was measured by the addition of *o*-nitrophenyl-D-galactopyranoside in phosphate-buffered saline with 5% Nonidet P-40. The resulting colorimetric reaction was measured in a spectrophotometric plate reader (Titertek, Huntsville, AL) at 420 nm. Data were plotted and analyzed using XLfit software (IDBS, Guildford, Surrey, U.K.).

Antagonist Assays for the D₂, 5-HT_{2B}, H₁ and alpha_{1A} receptors

Antagonist assays for the D₂, 5-HT_{2B}, H₁ and alpha_{1A} receptors were performed as described above, wherein, instead of transfecting cells with plasmid encoding human 5-HT_{2B} receptor DNA, the plasmid DNA for each gene of interest (i.e., D₂, 5-HT_{2B}, H₁ and alpha_{1A} receptors) was co-transfected with G_{q15}. For the 5-HT_{2B} receptor, no G-protein DNA was used. In order to assess the antagonist activity of each ligand, prior to addition of the cells to the 96 well plates containing the ligand under investigation, the cells were spiked with a known concentration of an appropriate agonist to stimulate receptor activity. The balance of the assay and data analysis was as explained for 5-HT_{2B} receptor inverse agonist assay above.

On certain occasions, cells were transfected with the gene of interest directly in 96 well plates. After 12-16 hours, medium was replaced with DMEM containing 1% PSG, 2% cyto-sf3 and varying concentrations of ligand under investigation. The balance of the assay was as described above for frozen cells. Assay activity results were found not to vary due to whether the cells used had been frozen or not.

EXHIBIT

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Exhibit 16

TABLE II: Activity at 5HT_{2B}, D2, H1 and Adrenergic Alpha 1A Receptors

Inverse agonist and antagonist activities are provided as averaged pIEC₅₀ or pK_i values derived from multiple experimental replicates (n) +/- standard deviation.

Example No.	Salt Form	5HT _{2B} Inverse Agonist Activity (pIEC ₅₀)	5HT _{2B} Antagonist Activity (pK _i)	Dopamine D2 Antagonist Activity (pK _i)	Histamine H1 Antagonist Activity (pK _i)	Adrenergic Alpha 1A Antagonist Activity (pK _i)
Example A	Tartrate	NA (n=3)	NA (n=3)	NA (n=4)	NA (n=3)	NA (n=3)
Example B	HCl	NA (n=3)	NA (n=3)	NA (n=6)	NA (n=4)	NA (n=3)
Example 1	Free Base	6.0 +/- 0.1 (n=2)				
Example 7	Free Base	6.3 +/- 0.3 (n=4)		6.9 (n=1)		
Example 8	Free Base	NA (n=4)				
Example 9	HCl	NA (n=3)				
Example 11	HCl	7.1 +/- 0.4 (n=6)	6.9 +/- 0.5 (n=3)	6.4 +/- 0.1 (n=2)		
Example 13	Free Base	6.2 +/- 0.3 (n=4)	6.7 (n=1)			
Example 13	HCl	6.3 +/- 0.3 (n=4)	6.8 (n=1)			
Example 14	Free Base	6.2 +/- 0.3 (n=5)				
Example 15	Free Base	6.1 +/- 0.3 (n=4)	6.6 +/- 0.2 (n=2)			
Example 15	HCl	6.6 +/- 0.1 (n=4)	7.3 (n=1)	6.6 +/- 0.3 (n=4)	6.5 +/- 0.5 (n=3)	
Example 16	Free Base	6.0 +/- 0.2 (n=3)	6.9 +/- 0.2 (n=2)			
Example 17	Free Base	6.0 +/- 0.2 (n=3)				
Example 18	Free Base	6.0 +/- 0.1 (n=2)				
Example 19	Free Base		6.6 (n=1)			
Example 20	Free Base	NA (n=1)				
Example 25	Free Base		6.4 (n=1)			
Example 29	Free Base	NA (n=2)				

Example No.	Salt Form	5HT _{2B} Inverse Agonist Activity (pIEC ₅₀)	5HT _{2B} Antagonist Activity (pK _i)	Dopamine D2 Antagonist Activity (pK _i)	Histamine H1 Antagonist Activity (pK _i)	Adrenergic Alpha 1A Antagonist Activity (pK _i)
Example 30	Free Base	NA (n=1)				
Example 35	Free Base	NA (n=1)				
Example 37	Free Base	NA (n=3)				
Example 38	Free Base	NA (n=3)				
Example 38	HCl	NA (n=1)				
Example 45	Free Base	6.2 +/- 0.3 (n=4)	6.7 (n=1)			
Example 45	HCl	6.3 +/- 0.3 (n=4)	6.8 (n=1)			
Example 46	HCl	6.9 +/- 0.2 (n=9)	6.8 +/- 0.3 (n=4)			
Example 48	HCl	6.8 +/- 0.5 (n=5)	7.0 +/- 0.0 (n=2)			
Example 52	HCl	6.2 +/- 0.3 (n=4)	6.6 +/- 0.1 (n=2)		6.6 (n=1)	
Example 53	HCl	6.0 +/- 0.3 (n=3)	6.6 +/- 0.1 (n=2)			
Example 54	HCl	6.2 (n=1)				
Example 55	HCl	6.0 (n=1)				
Example 56	HCl	NA (n=2)				
Example 58	HCl		6.9 (n=1)			
Example 59	HCl	NA (n=3)				
Example 60	HCl	6.1 +/- 0.3 (n=2)				
Example 61	HCl	NA (n=2)				
Example 63	HCl		NA (n=1)			
Example 68	TFA	NA (n=1)				
Example 68	HCl	NA (n=3)				
Example 69	TFA	6.3 +/- 0.2 (n=6)		7.0 +/- 0.4 (n=4)		
Example 69	HCl	6.3 +/- 0.2 (n=3)		7.1 +/- 0.1 (n=3)		

Example No.	Salt Form	5HT _{2B} Inverse Agonist Activity (pIEC ₅₀)	5HT _{2B} Antagonist Activity (pK _i)	Dopamine D2 Antagonist Activity (pK _i)	Histamine H1 Antagonist Activity (pK _i)	Adrenergic Alpha 1A Antagonist Activity (pK _i)
Example 70	HCl			6.4 +/- 0.0 (n=2)		
Example 71	HCl	NA (n=3)		6.8 +/- 0.3 (n=2)		
Example 72	HCl	NA (n=3)	NA (n=2)			
Example 73	HCl	NA (n=3)	NA (n=2)			
Example 74	HCl	6 +/- 0.2 (n=3)				
Example 75	HCl	6.5 +/- 0.3 (n=5)				
Example 76	TFA	7.4 (n=1)		7.6 +/- 0.4 (n=9)	6.2 +/- 0.2 (n=3)	6.6 (n=1)
Example 76	HCl	>6.7 (n=4)	>8.5 (n=2)	7.8 +/- 0.2 (n=4)		
Example 77	HCl	6.2 +/- 0.1 (n=3)				
Example 78	HCl	6.2 +/- 0.2 (n=3)		6.6 +/- 0.3 (n=4)		6.0 (n=1)
Example 79	TFA	NA (n=1)				
Example 80	TFA	NA (n=1)		6.2 (n=1)		
Example 81	TFA	6.0 +/- 0.1 (n=2)		6.5 +/- 0.0 (n=2)		
Example 82	TFA	NA (n=1)		6.7 +/- 0.3 (n=2)		
Example 83	TFA	NA (n=2)		6.5 +/- 0.5 (n=4)		
Example 84	HCl	NA (n=2)		6.7 +/- 0.4 (n=6)	6.0 +/- 0.8 (n=3)	NA (n=1)
Example 85	HCl	6.0 +/- 0.2 (n=2)		6.2 +/- 0.3 (n=3)		
Example 87	Free Base	NA (n=2)				
Example 88	Free Base	NA (n=2)				
Example 89	HCl	NA (n=3)		6.5 +/- 0.9 (n=3)	NA (n=1)	6.0 (n=1)
Example 90	HCl	NA (n=2)		6.4 +/- 0.2 (n=3)		
Example 91	HCl	6.5 +/- 0.4 (n=4)			NA (n=1)	NA (n=1)
Example 92	HCl	6.1 +/- 0.4 (n=3)		6.3 +/- 0.4 (n=2)		

Example No.	Salt Form	5HT _{2B} Inverse Agonist Activity (pIEC ₅₀)	5HT _{2B} Antagonist Activity (pK _i)	Dopamine D2 Antagonist Activity (pK _i)	Histamine H1 Antagonist Activity (pK _i)	Adrenergic Alpha 1A Antagonist Activity (pK _i)
Example 93	HCl	NA (n=3)		6.3 +/- 0.1 (n=3)	6.7 (n=1)	
Example 94	HCl	6.0 (n=1)				
Example 95	HCl	6.0 +/- 0.2 (n=2)				
Example 96	HCl	6.5 +/- 0.3 (n=2)		6.9 (n=1)		
Example 97	di-HCl	NA (n=2)	NA (n=2)			
Example 98	HCl	6.1 +/- 0.1 (n=3)				
Example 99	HCl	NA (n=2)				
Example 100	HCl	6.4 +/- 0.5 (n=4)	7.0 +/- 0.0 (n=2)			
Example 101	HCl	6.2 +/- 0.1 (n=4)	6.9 +/- 0.1 (n=2)			
Example 102	HCl	6.2 +/- 0.3 (n=4)	6.8 +/- 0.2 (n=2)			
Example 103	HCl	6.1 +/- 0.1 (n=4)	6.8 +/- 0.1 (n=2)			
Example 104	HCl	NA (n=5)				
Example 105	HCl		6.6 (n=1)			
Example 106	HCl	NA (n=1)				
Example 107	Oxalate	NA (n=3)				
Example 108	Oxalate	6.7 +/- 0.3 (n=6)				
Example 109	Oxalate	6.2 +/- 0.1 (n=3)				
Example 110	Oxalate	6.2 +/- 0.2 (n=3)				
Example 111	HCl	NA (n=2)			7.1 (n=1)	
Example 112	HCl			6.7 +/- 0.3 (n=2)		
Example 113	HCl	NA (n=1)				
Example 114	HCl	NA (n=1)				
Example 115	HCl	NA (n=1)				

Example No.	Salt Form	5HT _{2B} Inverse Agonist Activity (pIEC ₅₀)	5HT _{2B} Antagonist Activity (pK _i)	Dopamine D2 Antagonist Activity (pK _i)	Histamine H1 Antagonist Activity (pK _i)	Adrenergic Alpha 1A Antagonist Activity (pK _i)
Example 116	HCl	6.1 +/- 0.0 (n=2)				
Example 117	HCl	NA (n=2)				
Example 118	HCl	NA (n=1)				
Example 119	HCl	NA (n=3)	NA (n=1)			
Example 120	HCl		6.2 (n=1)			
Example 124	HCl	6.1 (n=1)				
Example 125	HCl	6.1 +/- 0.2 (n=2)				
Example 126	HCl	NA (n=3)	NA (n=1)			
Example 127	HCl	6.3 +/- 0.1 (n=3)			6.6 +/- 0.2 (n=2)	
Example 128	HCl	6.0 +/- 0.1 (n=2)				
Example 129	HCl	6.0 +/- 0.1 (n=2)				
Example 130	HCl	6.1 +/- 0.0 (n=2)				
Example 131	HCl	6.0 (n=1)		7.0 (n=1)	6.9 +/- 0.3 (n=2)	
Example 132	HCl			6.5 +/- 0.5 (n=3)	6.1 (n=1)	
Example 133	HCl	6.3 +/- 0.0 (n=2)		6.3 (n=1)		
Example 134	HCl	6.6 +/- 0.1 (n=3)		6.5 +/- 0.8 (n=2)	NA (n=1)	NA (n=1)
Example 135	HCl	NA (n=2)		6.5 +/- 0.3 (n=2)	6.2 (n=1)	
Example 136	HCl	6.1 +/- 0.0 (n=2)		6.6 +/- 1.0 (n=3)	6.1 +/- 1.3 (n=2)	NA (n=1)
Example P-2 ¹	HCl	6.1 +/- 0.4 (n=2)				
Example P-3	HCl	6.1 +/- 0.0 (n=2)		6.0 (n=1)		
Example P-4	HCl	6.3 +/- 0.2 (n=2)		6.3 +/- 0.1 (n=2)		

¹ Refers to compounds recited in Andersson at pages 25-27.

Example No.	Salt Form	5HT _{2B} Inverse Agonist Activity (pIEC ₅₀)	5HT _{2B} Antagonist Activity (pK _i)	Dopamine D2 Antagonist Activity (pK _i)	Histamine H1 Antagonist Activity (pK _i)	Adrenergic Alpha 1A Antagonist Activity (pK _i)
Example P-5	HCl	NA (n=1)				
Example P-6	HCl	6.3 +/- 0.2 (n=3)				
Example P-7	HCl	NA (n=1)				
Example P-8	HCl	6.6 +/- 0.6 (n=3)		6.4 +/- 0.2 (n=2)		
Example P-9	HCl	7.9 +/- 0.5 (n=2)		7.0 +/- 0.2 (n=4)	NA (n=1)	6.6 (n=1)
Example P-10	HCl	NA (n=2)		6.4 (n=1)		
Example P-11	HCl	NA (n=1)		NA (n=1)	NA (n=1)	NA (n=1)
Example P-12	HCl	NA (n=3)				

EXHIBIT

17

Research Paper

Prediction of Human Drug Clearance from *in Vitro* and Preclinical Data Using Physiologically Based and Empirical Approaches

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Purpose. The aim of this study is to compare the accuracy of five methods for predicting *in vivo* intrinsic clearance (CL_{int}) and seven for predicting hepatic clearance (CL_h) in humans using *in vitro* microsomal data and/or preclinical animal data.

Methods. The human CL_{int} was predicted for 33 drugs by five methods that used either *in vitro* data with a physiologic scaling factor (SF), with an empirical SF, with the physiologic and drug-specific (the ratio of *in vivo* and *in vitro* CL_{int} in rats) SFs, or rat CL_{int} directly and with allometric scaling. Using the estimated CL_{int} , the CL_h in humans was calculated according to the well-stirred liver model. The CL_h was also predicted using additional two methods: using direct allometric scaling or drug-specific SF and allometry.

Results. Using *in vitro* human microsomal data with a physiologic SF resulted in consistent underestimation of both CL_{int} and CL_h . This bias was reduced by using either an empirical SF, a drug-specific SF, or allometry. However, for allometry, there was a substantial decrease in precision. For drug-specific SF, bias was less reduced, precision was similar to an empirical SF. Both CL_{int} and CL_h were best predicted using *in vitro* human microsomal data with empirical SF. Use of larger data set of 52 drugs with the well-stirred liver model resulted in a best-fit empirical SF that is 9-fold increase on the physiologic SF.

Conclusions. Overall, the empirical SF method and the drug-specific SF method appear to be the best methods; they show lower bias than the physiologic SF and better precision than allometric approaches. The use of *in vitro* human microsomal data with an empirical SF may be preferable, as it does not require extra information from a preclinical study.

KEY WORDS: allometry; clearance prediction; hepatic clearance; intrinsic clearance, *in vitro* scaling.

INTRODUCTION

Several approaches have been advocated to predict drug clearance in humans that involve the use of *in vitro* human microsomal data and/or preclinical animal data. *In vitro* drug metabolism kinetic parameters can provide an estimate of *in vivo* intrinsic clearance (CL_{int}) for the whole liver by the use of a scaling factor (SF) and subsequently hepatic clearance (CL_h) with the use of a liver model (1). Physiologically based

scaling factors (PB-SF) are preferred, for example, hepatocellularity for isolated hepatocytes, and for microsomes a SF accounting for incomplete microsomal recovery from human liver tissue based on CYP content in homogenate and microsomes (1–3). Alternative approaches to the use of *in vitro* data have also been suggested for the prediction of human drug clearances that use preclinical animal data; these include the use of allometry (4–8), drug-specific factors based on rat *in vitro* and *in vivo* parameters (9), as well as considering a combination of both of the above (10,11). In addition, some of the investigators have suggested the possibility of ignoring drug binding within the plasma and microsomal matrices on the grounds that, if identical, these parameters will cancelled out when liver models are applied (7).

The aim of this study was to investigate the full spectrum of these different approaches and make direct comparisons of their accuracy and precision for predicting human *in vivo* clearances with a much larger data set ($n = 52$) than that used in previous studies (3,7,11). *In vitro* CL_{int} data were taken from published human and rat microsomal studies while comparable *in vivo* CL_{int} were calculated via the well-stirred liver model from published CL_h data. Data for 33 drugs were used to compare five different approaches for predicting CL_{int} and seven approaches for predicting CL_h . PB approaches are compared to several empirical approaches using regression analysis, allometry, or preclinical *in vivo* data either alone or

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ABBREVIATIONS: *afe*, average fold-error; B_h , mean body weights of humans; B_r , mean body weights of rats; $CL_{h,h}$, hepatic clearance in humans; $CL_{h,r}$, hepatic clearance in rats; CL_{int} , intrinsic clearance; CL_h , hepatic clearance; $CL_{int,h,in vitro}$, *in vitro* intrinsic clearance in humans; $CL_{int,h,in vivo}$, *in vivo* intrinsic clearance in humans; $CL_{int,r,in vitro}$, *in vitro* intrinsic clearance in rats; $CL_{int,r,in vivo}$, *in vivo* intrinsic clearance in rats; *fup*, plasma unbound fraction; *fu_m*, unbound fraction in microsomes; *K_a*, affinity constant for the protein; *K_{a,m}*, affinity constant for drug binding in microsomes; *K_{a,p}*, affinity constant for drug binding in plasma; *mse*, mean squared prediction error; *P*, protein concentration; PB-SF, physiologically based SF; Q_h , hepatic blood flow; R_B , blood-to-plasma concentration ratio; *rmse*, root mean squared prediction error; SF, scaling factor.

in combination with *in vitro* data for specific drugs. For CL_h estimation, direct prediction and prediction via the use of CL_{int} and a liver model is compared. In addition, the consequences of nonspecific drug binding is investigated, both in the incorporation of plasma binding in liver models and the impact of relative drug binding within plasma and microsomal matrices using data for 46 drugs.

MATERIALS AND METHODS

Data Collection

In vitro intrinsic clearance in humans ($CL_{int,h,in vitro}$) was obtained from published metabolic studies using human liver microsomes. *In vivo* intrinsic clearance in humans ($CL_{int,h,in vivo}$) was calculated from the published values of CL_h , plasma unbound fraction (f_u,p), and blood-to-plasma concentration ratio (R_B) for each drug according to the well-stirred liver model as follows:

$$CL_{int,h,in vivo} = Q_h \times CL_h / (Q_h - CL_h) / (f_u,p / R_B) \quad (1)$$

where Q_h represents the hepatic blood flow (20.7 ml min⁻¹ kg⁻¹). These values for humans were available for 52 drugs (see Table I).

The *in vitro* and *in vivo* intrinsic clearances in rats ($CL_{int,r,in vitro}$ and $CL_{int,r,in vivo}$, respectively) were also obtained for 33 drugs (no. 1-33 in Table I) in a similar manner as those for humans except that Q_h of 100 (ml min⁻¹ kg⁻¹) was used.

For the human data, *in vivo* intrinsic clearances were also calculated using the assumptions of the parallel-tube liver model as follows:

$$CL_{int,h,in vivo} = -Q_h / (f_u,p / R_B) \times \ln(1 - CL_h / Q_h) \quad (2)$$

The assumptions of both the well-stirred and parallel-tube liver models have been previously discussed (27). Blood flow estimates were based on published values for human (28,29) and rat (30,31). It should be noted that the higher the clearance value, the more sensitive the CL_{int} calculation is to the blood flow value used.

Prediction of the *in Vivo* Intrinsic Clearance

$CL_{int,h,in vivo}$ (ml min⁻¹ kg⁻¹) for the 33 drugs for which rat data were available was predicted using the following five methods (A-E) and compared with the observed CL_{int} values calculated from CL_h as described above.

A. Using a Physiologically Based Scaling Factor

This method uses human microsomal data and a physiologically based scaling factor (PB-SF) based on hepatic microsomal recovery from the whole liver for conversion of the unit of the CL_{int} from ml min⁻¹ mg⁻¹ protein to ml min⁻¹ kg⁻¹ (1).

$$CL_{int,h,in vivo} = CL_{int,h,in vitro} (\text{ml/min/mg protein}) \times \text{PB-SF} \quad (3)$$

where the PB-SF (856 ± 270 mg protein/kg) is the average recovery ± SD of microsomal protein per gram of liver (40 mg protein/g liver) determined using 38 human livers (Hakooz *et al.*, unpublished observation) multiplied by the average liver weight in humans (21.4 g liver/kg).

B. Using an Empirical Scaling Factor

This method uses human microsomal data and an empirical SF of 6.2 (SD 0.2)g protein/kg body weight as a substitute for PB-SF and takes into account the extent of under-prediction associated with using method A. The empirical SF was determined by the regression analysis to obtain the best fit of $CL_{int,h,in vivo}$ and $CL_{int,h,in vitro}$ values using an equation analogous to Eq. 3. The leave-one-out approach was used and the average coefficient taken as the empirical SF.

C. Physiologically Based and Drug-Specific Scaling Factors

Like method A, this approach uses human microsomal data and PB-SF plus a drug specific factor based on *in vivo* and *in vitro* CL_{int} in rats (both in the unit of ml min⁻¹ kg⁻¹) (9).

$$CL_{int,h,in vivo} = CL_{int,h,in vitro} \times \text{PB-SF} \times (CL_{int,r,in vivo} / CL_{int,r,in vitro}) \quad (4)$$

where $CL_{int,r,in vitro}$ (ml min⁻¹ kg⁻¹) was calculated using the scaling factor of 500 (mg protein/250 g) (2).

D. Rat Intrinsic Clearance

No scaling factors are used in this approach, only *in vivo* rat data (ml min⁻¹ kg⁻¹).

$$CL_{int,h,in vivo} = CL_{int,r,in vivo} \quad (5)$$

E. Using Allometric Scaling

As in D, this method uses rat *in vivo* data but also involves an allometric factor (4,5).

$$CL_{int,h,in vivo} = CL_{int,r,in vivo} \times (B_h / B_r)^x \quad (6)$$

where B_h and B_r are the mean body weights of humans (70 kg) and rats (250 g), respectively. The exponent (x) for each individual drug was determined by Eq. 7, which is re-arranged from Eq. 6 using the observed values of $CL_{int,h,in vivo}$.

$$x = \log(CL_{int,h,in vivo} / CL_{int,r,in vivo}) / \log(B_h / B_r) \quad (7)$$

The average exponent (x) for all drugs excluding the drug under study is then used for the allometric scaling of individual drugs ($x = 0.538 \pm 0.293$).

Prediction of the Hepatic Clearance

Using the *in vivo* intrinsic clearance calculated above, the hepatic clearance in humans ($CL_{h,h}$) was calculated (methods A-E) according to the "well-stirred" liver model as follows:

$$CL_{h,h} = Q_h \times f_u,p / R_B \times CL_{int,h,in vivo} / (Q_h + f_u,p / R_B \times CL_{int,h,in vivo}) \quad (8)$$

where Q_h of 20.7 (ml min⁻¹ kg⁻¹) was used as above.

The $CL_{h,h}$ was also directly calculated using the following two methods.

F. Direct Allometric Scaling

For this approach, rat hepatic clearance ($CL_{h,r}$) data are used directly with an allometric factor (8).

$$CL_{h,h} = CL_{h,r} \times (B_h / B_r)^y \quad (9)$$

Table I. Values of Intrinsic Clearance in Humans and Rats

No.	Drugs ^a	Human CL _{int} (ml min ⁻¹ kg ⁻¹)		Rat CL _{int} (ml min ⁻¹ kg ⁻¹)	
		<i>in vitro</i>	<i>in vivo</i>	<i>in vitro</i>	<i>in vivo</i>
1	FK1052 ⁹	40	1600	1100	28,000
2	FK480 ⁹	51	340	250	5700
3	Zolpidem ⁹	20	160	170	730
4	Omeprazole ⁹	67	520	650	5700
5	Nicardipine ⁹	1200	1900	13,000	14,000
6	Nilvadipine ⁹	1200	8400	72,000	240,000
7	Diazepam ⁹	10	21	500	760
8	Diltiazem ⁹	81	300	730	1600
9	Diazepam ²	4.1	13	220	1300
10	Midazolam	44	270	220	1000
11	Triazolam	13	38	340	1600
12	Flunitrazepam	5.0	11	240	380
13	Alprazolam	2.0	3.1	470	1300
14	Phenytoin ^{2,12}	0.16	4.0	92	220
15	Tolbutamide ^{2,12}	1.2	2.0	16	6.0
16	Ibuprofen ^{1,12}	8.2	83	77	240
17	Diclofenac ^{12,13}	35	630	280	670
18	Imipramine ^{1,14}	18	310	9000	7500
19	Warfarin ^{2,14}	0.49	4.5	9.1	20
20	Hexobarbital ^{1,14}	2.2	8.2	870	320
21	Dofetilide ^{2,3}	0.40	4.5	36	96
22	Metoprolol ^{1,3}	18	26	88	680
23	Phenacetin ^{1,3}	19	46	66	520
24	s-Warfarin ^{2,3}	1.0	5.7	4.2	16
25	r-Warfarin ^{2,3}	0.15	5.4	14	24
26	YW796 ¹⁵	15	14	1700	4800
27	Indinavir ¹⁶⁻¹⁸	16	130	140	570
28	Lidocaine ^{1,8,19}	3.1	55	400	2500
29	Ondansetron ^{2,8,20}	1.7	33	850	630
30	Antipyrine ^{1,11,21}	0.14	0.51	7.2	6.0
31	Caffeine ^{2,11,22}	0.43	3.5	11	14
32	Felodipine ^{1,11,23}	98	4300	1000	440
33	Propranolol ^{11,24-26}	90	340	8600	41000
34	Chlorpromazine ¹⁴	24	370		
35	Propafenone ¹⁴	160	4000		
36	Verapamil ¹⁴	120	1800		
37	Diphenhydramine ¹⁴	2.0	52		
38	Lorcanide ¹⁴	48	710		
39	Amitriptyline ¹⁴	13	490		
40	Desipramine ¹⁴	16	150		
41	Ketamine ¹⁴	26	550		
42	Quinidine ¹⁴	3.2	22		
43	Clozapine ¹⁴	4.4	59		
44	Dexamethasone ¹⁴	2.9	14		
45	Prednisone ¹⁴	2.6	21		
46	Methoxsalen ¹⁴	38	1000		
47	Tenidap ¹⁴	7.9	80		
48	Tenoxicam ¹⁴	1.6	2.2		
49	Amobarbital ¹⁴	0.89	1.4		
50	Methohexital ¹⁴	47	180		
51	Mexiletine ³	0.77	26		
52	Theophylline ³	0.033	3.5		

^a Reference source given after drug name.

The exponent (y) for each individual drug was determined by Eq. 10, which is re-arranged from Eq. 9 using the observed values of CL_{h,h}.

$$y = \log (CL_{h,h}/CL_{h,r})/\log (B_h/B_r) \quad (10)$$

The average exponent (y) for all drugs excluding the drug

under study is then used for the allometric scaling of individual drugs ($y = 0.616 \pm 0.190$).

G. A Drug-Specific Scaling Factor and Allometry

This method uses rat hepatic clearance data directly with both an allometric factor and the drug specific factor outlined in method C (10,11).

$$CL_{h,h} = CL_{h,r} \times (CL_{int,h,in vitro} / CL_{int,r,in vitro}) \times (B_h / B_r)^z \quad (11)$$

The exponent (z) for each individual drug was determined by Eq. 12, which is re-arranged from Eq. 11 using the observed values of $CL_{h,h}$.

$$z = \log (CL_{h,h} / CL_{h,r} \times CL_{int,r,in vitro} / CL_{int,h,in vitro}) / \log (B_h / B_r) \quad (12)$$

The average exponent (z) for all drugs excluding the drug under study is then used for the allometric scaling of individual drugs ($z = 1.23 \pm 0.23$). The predicted $CL_{h,h}$ values were replaced by the value of Q_h (20.7 ml/min/kg) when they were above Q_h (5 out of 33 cases).

Accuracy of Predictions

The accuracy of the predictions was assessed from the prediction error (difference between predicted and observed *in vivo* values) for each drug in a particular *in vitro* system. For visual inspection this was plotted as the log of the ratio of predicted/observed clearance against the predicted clearance (2) and the limits of ± 0.3 represented a 2-fold error. For the $CL_{int,h,in vivo}$, the predicted/observed clearance ratio was calculated directly and for CL_h predictions, the error in CL_{int} was propagated through the liver model calculation. Both 2- and 3-fold error limits were propagated.

Also the accuracy of each prediction method was compared from the root mean squared prediction error (*rmse*) and the average fold-error (*afe*) as measures of precision and bias, respectively, estimated as follows (7,32):

$$afe = 10^{\left| \frac{1}{N} \sum \log \frac{\text{Predicted}}{\text{Observed}} \right|} \quad (13)$$

$$mse = \frac{1}{N} \sum (\text{Predicted} - \text{Observed})^2, \quad rmse = \sqrt{mse} \quad (14)$$

The variance of the prediction is calculated from the sum of the squares of the prediction errors and this provides the *rmse*. The geometric mean of the prediction error provides a measure of bias with equal value to under- and over-predictions in the form of *afe*.

The correlation analyses were also performed between the predicted and observed values for each parameter to obtain the squared correlation coefficient (r^2).

Impact of Ignoring Protein Binding

In *in vitro* liver microsomal studies, some drugs bind non-specifically within the matrix, hence the kinetic parameters estimated need to be corrected to reflect the unbound drug. On the other hand, in the equations for liver models, drug binding in blood and microsomes would theoretically cancel out if the unbound fraction (*fu*) is the same for blood and microsomes (7):

$$CL_h = Q_h \times fu,p / R_B \times (CL_{int} / fu,m) / (Q_h + fu,p / R_B \times (CL_{int} / fu,m)) \quad (15)$$

where *fu,m* is the unbound fraction in microsomes.

The validity of this assumption was investigated by the following two approaches. Firstly, the human $CL_{int,h,in vivo}$ values for the above-mentioned 52 drugs were calculated according to the Eq. 16, modified from Eq. 1, ignoring the plasma protein binding:

$$CL_{int,h,in vivo} = Q_h \times CL_h / (Q_h - CL_h) \quad (16)$$

The calculated values of $CL_{int,h,in vivo}$ were then compared with the values predicted using the PB-SF (Eq. 3).

In the second approach, the *fu* for both human plasma and human liver microsomes were collected from literature (9,14,33) or obtained in our laboratory (unpublished observation). 46 drugs in total were classified into basic, neutral, and acidic drugs, depending on their charge at physiologic pH. The *fu* of a drug can be expressed by the following equation, using the affinity constant for tissue macromolecules, e.g., protein, (K_a) and tissue macromolecules (binding site) concentration (P):

$$fu = 1 / (1 + K_a P) \quad (17)$$

In plasma the average albumin concentration is 40 mg/ml. The *fu,m* values are normally measured at the microsomal concentration used in the metabolism study, which differs with substrate used and laboratory. Therefore for each drug the *fu,m* at the protein concentration of 1 mg/ml was estimated using Eq. 17 and using the reported values of P . If the K_a for microsomes ($K_{a,m}$) is assumed to be proportional to that for plasma ($K_{a,p}$) ($K_{a,m} / K_{a,p} = a$), the equation for *fu,p* at $p = 40$ mg/ml and that for *fu,m* at $p = 1$ mg/ml can be combined to give the following equation:

$$fu,m = 40 fu,p / \{a + (40 - a) fu,p\} \quad (18)$$

The relationship between *fu,m* and *fu,p* was analyzed based on Eq. 18.

RESULTS

Prediction of the *in Vivo* Intrinsic Clearance

Figures 1A–1E illustrate the correlations between the observed and predicted values of $CL_{int,h,in vivo}$ using each of the five methods. In order to assess the accuracy and bias of the predictions, the prediction error (expressed as the log of the predicted/observed clearance ratio) were plotted as a function of predicted clearance (Fig. 2) and various statistical parameters calculated (Table II).

Human microsomal data in combination with the PB-SF (method A) resulted in a strong correlation ($r^2 = 0.82$) but a general underprediction of CL_{int} (Fig. 1A). The bias in this method was reduced by the introduction of either an empirical SF (method B) or a drug-specific factor (the ratio of *in vivo* and *in vitro* CL_{int} obtained in rats—method C), see Table II. There is no bias when rat *in vivo* data are used with the allometric factor (method E) which is marked better than using rat *in vivo* data alone (method D). However method E, in comparison to methods B and C, is associated with the poorest precision and the lowest r^2 of these predictions (Fig. 1E, Table II) when considered relative to the PB-SF (method A).

Prediction of the Hepatic Clearance

CL_h were predicted using the well-stirred liver model and the $CL_{int,h,in vivo}$ values calculated from each of the five methods (A–E) described above. In addition two other methods (F and G) were also used to predict CL_h directly, either using allometry alone or in combination with a drug specific factor. Graphical comparisons of the prediction methods are

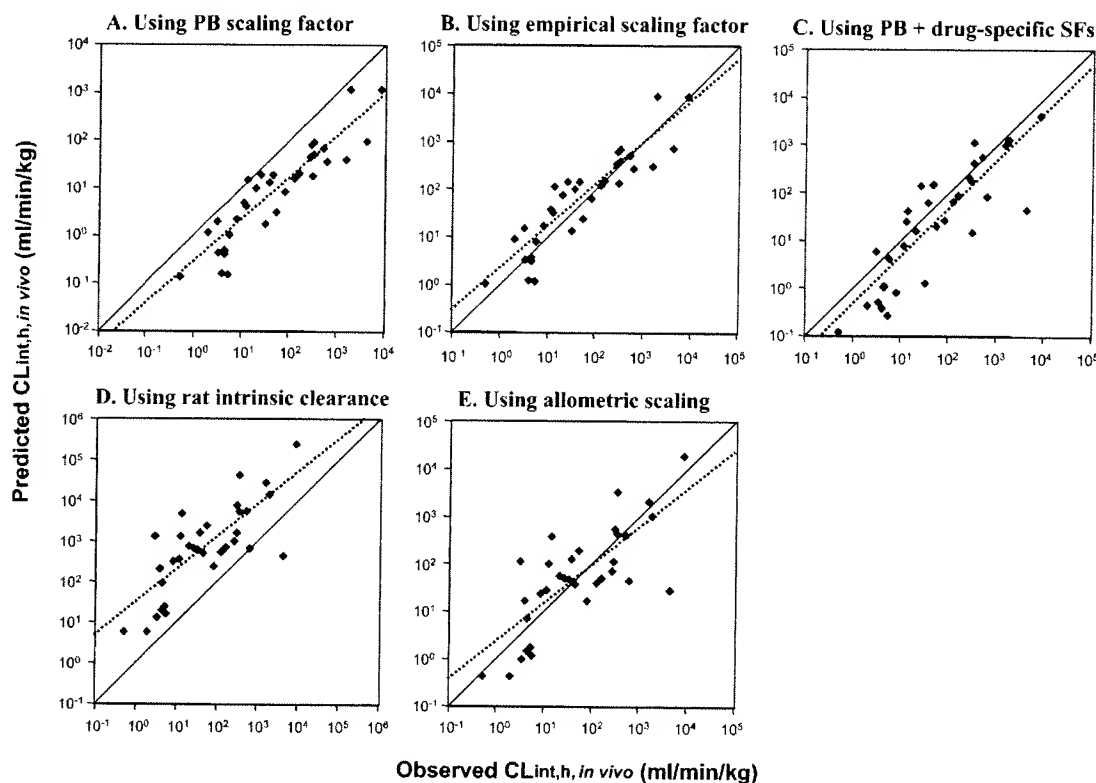


Fig. 1. Correlation between the observed and predicted human CL_{int} for 33 drugs determined using the five different approaches. (A) Using PB-SF, (B) using empirical SF, (C) using PB-SF and drug-specific SF, (D) rat intrinsic clearance, and (E) using allometric scaling. Lines represent the regression (dotted line) and unity (solid line).

shown in Fig. 3 and the parameters for the accuracy of predictions are summarized in Table II.

Similar trends for CL_h predictions for methods A–E were seen to that discussed above for CL_{int} (see Fig. 3, method D not shown). Prediction of CL_h directly by allometry (method F, Fig. 3F) showed low bias but poor precision and a particularly low r^2 . Incorporation of a drug specific factor (method G, Fig. 3G) did little to improve this approach. If Q_h did not restrict the predicted CL_h values (i.e. if the values above 20.7 ml/min/kg were allowed, 5 out of the 33 cases) in method G, the value of $rmse$ is increased to 19.2 and the r^2 is reduced to 0.154. These characteristics are clearly illustrated in the precision error for CL_h as shown in Fig. 4.

Extension of the Database

It is possible to extend the human database (but without the corresponding rat parameters) for an additional 19 drugs further assess method B with CL_{int} values for 52 drugs. These data were used to predict CL_{int} using the PB-SF (method A) and compared to CL_{int} calculated from *in vivo* data using either the well-stirred or the parallel tube liver model (Figs. 5A and 5B). Both observed and predicted values for the well-stirred model are also listed in Table I. Regression analysis using either the well-stirred or the parallel tube liver models, respectively, generated empirical scaling factors of 7.9 g protein/kg and 5.4 g protein/kg. Thus the extent of underestimation apparent with the use of method A to predict *in vivo* CL_{int} is approximately 9- and 6-fold, depending on the liver model used.

Effects of Plasma Protein Binding

Figure 5C shows the prediction of human CL_{int} for 52 drugs without incorporating plasma protein binding (using the well-stirred model). Compared with the prediction using PB-SF with plasma protein binding incorporated (Fig. 5A), the bias was reduced by ignoring the plasma protein binding, but the precision was also reduced.

Figure 6 compares nonspecific drug binding in plasma and microsomal matrices, the $f_{u,m}$ at a microsomal protein concentration of 1 mg/ml is plotted against the $f_{u,p}$ (plasma protein concentration 40 mg/ml). The lines shown are based on Eq. 18 and either assume equality in binding ($K_{a,m}/K_{a,p}$ ratio of unity) or refer to the $K_{a,m}/K_{a,p}$ ratio obtained by regression analysis. Only for neutral drugs were K_a values similar between microsomes and plasma ($K_{a,m}/K_{a,p}$ ratio = 1.02). For basic drugs and acidic drugs the $K_{a,m}$ was higher (average $K_{a,m}/K_{a,p}$ ratio 8.7) or lower (average $K_{a,m}/K_{a,p}$ ratio 0.022), respectively.

DISCUSSION

In order to assess various approaches to predicting human *in vivo* clearance a data base of 52 drugs has been collected from a variety of literature sources. Comparisons published previously have focused on a relatively small number of drugs (3,7,11) and have been less comprehensive in the range of approaches investigated. The present analysis considers primarily *in vitro* and *in vivo* CL_{int} values, the latter obtained from “deconstructing” CL_h to generate a wide range of parameter values (4 orders of magnitude) to allow detailed comparisons. Also considered is the prediction of CL_h .

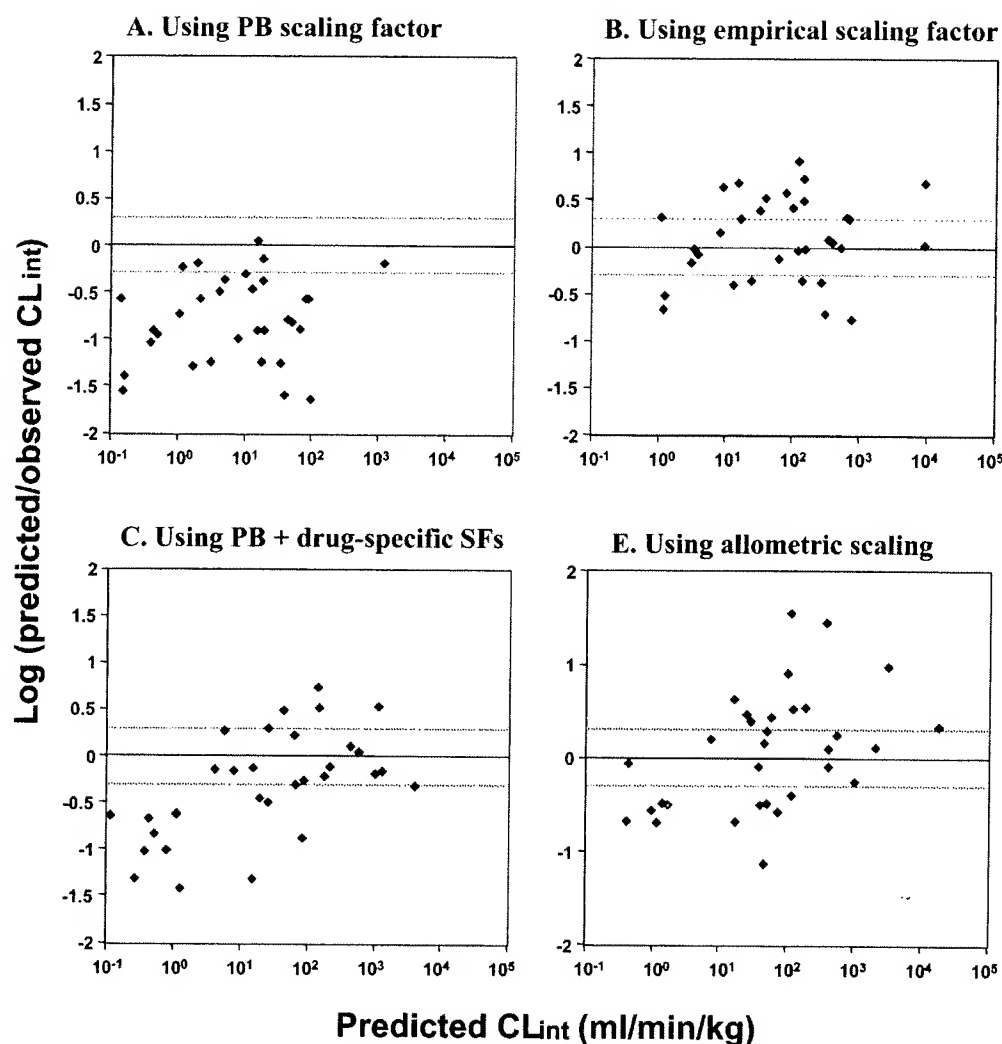


Fig. 2. Precision error (expressed as the log of the predicted/observed CL_{int} ratio) for predicted CL_{int} for 33 drugs using four different approaches. (A) Using PB-SF, (B) using empirical SF, (C) using PB-SF and drug-specific SF, and (E) using allometric scaling. The corresponding panel for method D has been excluded as the data were off-scale. Lines represent the limits of a 2-fold error (± 0.3 log unit) in the predicted value.

The five approaches investigated to predict CL_{int} use kinetic parameters from *in vitro* human tissues, *in vivo* and *in vitro* preclinical studies or a combination of both. The human hepatic microsomal CL_{int} parameters show an excellent correlation with the corresponding *in vivo* data (Figs. 1A and B) and a marked improvement over the correlation seen with

preclinical (rat) data with or without allometry (Figs. 1D and E). Combination of preclinical and human *in vitro* data has the advantage of providing a drug specific factor that would theoretically correct for any systematic difference between *in vitro* and *in vivo* parameters that is peculiar to that particular drug, such as microsomal binding. However as illustrated in

Table II. Statistical Data Comparing the Accuracy of Predictions Using Different Methods

		Methods						
		A	B	C	D	E	F	G
CL_{int}	<i>afe</i>	6.17	1.00	2.33	13.48	1.00		
	<i>rmse</i>	1500	1245	1099	41945	1993		
	r^2	0.820	0.809	0.736	0.602	0.583		
	% outside 2-fold error	84.8	54.5	60.6	97.0	69.7		
CL_h	<i>afe</i>	4.52	1.09	2.23	3.01	1.19	1.00	1.12
	<i>rmse</i>	5.35	3.71	3.89	9.24	4.77	5.31	6.69
	r^2	0.587	0.675	0.654	0.390	0.517	0.224	0.322

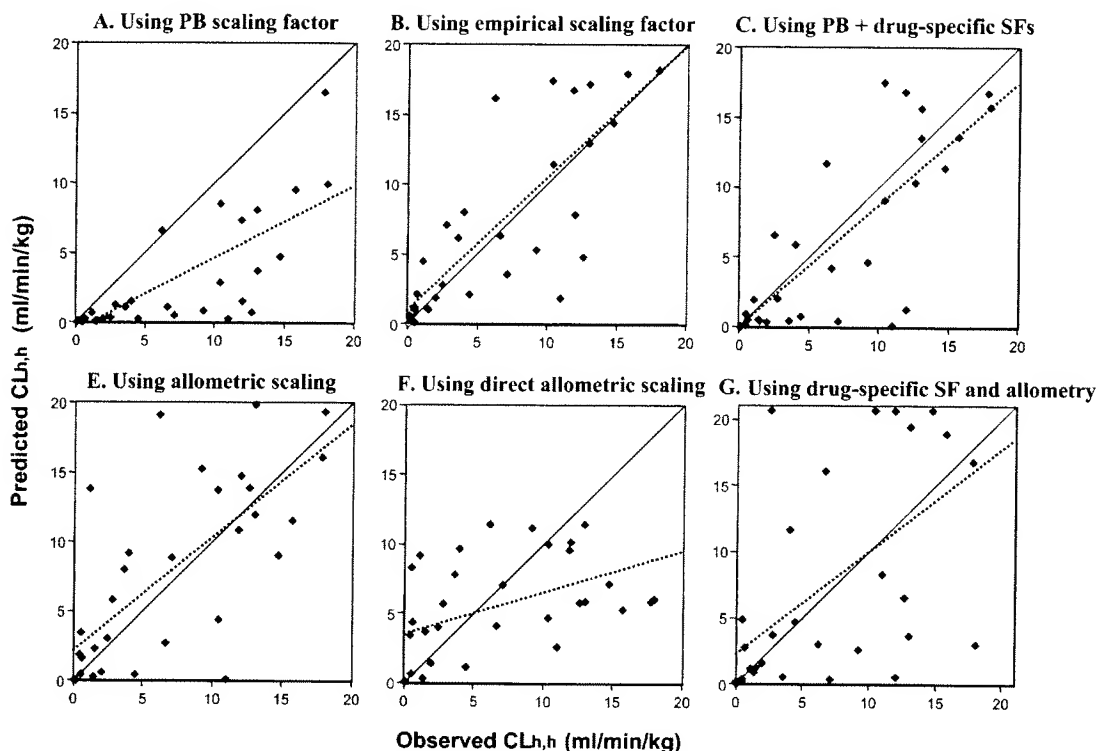


Fig. 3. Correlations between the observed and predicted human CL_h for 33 drugs determined using six different approaches. (A) Using PB-SF, (B) using empirical SF, (C) using PB-SF and drug-specific SF, (E) using allometric scaling, (F) direct allometric scaling, and (G) Using drug-specific SF and allometry. Lines represent the regression (dotted line) and unity (solid line).

Fig. 1C, it provided no improvement in the precision of the prediction over the use of human *in vitro* data alone.

A similar picture was obtained from consideration of CL_h prediction using the five approaches to determining CL_{int} and the well-stirred liver model. Human microsomal derived CL_{int} again provided the most accurate predictions of CL_h . The two further methods were investigated for CL_h prediction, using allometry from rat CL_h directly as well as in combination with a drug-specific factor. Once again, neither of these options provided an improvement on the use of *in vitro* microsomal parameters.

The bias of a prediction is of importance as well as the precision. Using the PB approach (based on microsomal enzyme recovery) to scale *in vitro* CL_{int} to provide *in vivo* CL_{int} values results in a systematic underprediction. The selection of the particular liver model used to "deconstruct" CL_h to give an *in vivo* CL_{int} influences the extent to which this occurs. This is because the parallel tube model always gives lower *in vivo* CL_{int} values than the well-stirred liver model and the dispersion model gives intermediate values (34). In a recent analysis of the use of liver models for prediction of rat clearances from both hepatocytes and hepatic microsomes it was concluded that there was the minimal differences between the liver models. Thus the simplest, most commonly adopted, well-stirred liver model could continue to be used with confidence (34).

The bias seen with *in vitro* data can be reduced or removed by a number of alternative procedures. Overall the use of an empirical scaling factors (method B) and the drug-specific method (method C) appear to be the best methods, as

they show lower bias than the PB scaling factor and better precision than the allometric approaches. The use of *in vitro* human microsomal data with an empirical scaling factor (method B) may be regarded as preferable, as it does not require extra information from preclinical studies both *in vitro* and *in vivo*.

The decision of whether to incorporate plasma protein binding in clearance prediction is controversial. Whereas a basic tenet of physiologically based pharmacokinetics is that the unbound drug concentration in the plasma dictates tissue distribution, there have been reports that *in vitro* clearance provides a better estimate of *in vivo* clearance of total rather than unbound drug concentration (7,35,36). Ignoring plasma binding in the present analysis provides a good example of how the bias can be removed from a prediction but only at the expense of a loss in precision. A further variant on this idea is the relative role of drug binding in plasma and hepatic microsomes. It is an attractive option (7) to consider that the parameters describing the two processes may cancel out in the liver model calculation, and hence neither process needs to be measured. However it is clear from the comparative analysis of microsomal and plasma binding presented here that drug binding within these two matrices is unlikely to be equivalent and hence should not be cancelled out in principle.

The fraction unbound in either matrix is dependent upon protein/lipid (binding sites) concentration and K_a values. For plasma, albumin is usually the major site of drug binding but within the microsomal matrix the high content of lipid provides potential binding sites in addition to protein. In the analysis presented here protein is taken as the common bind-

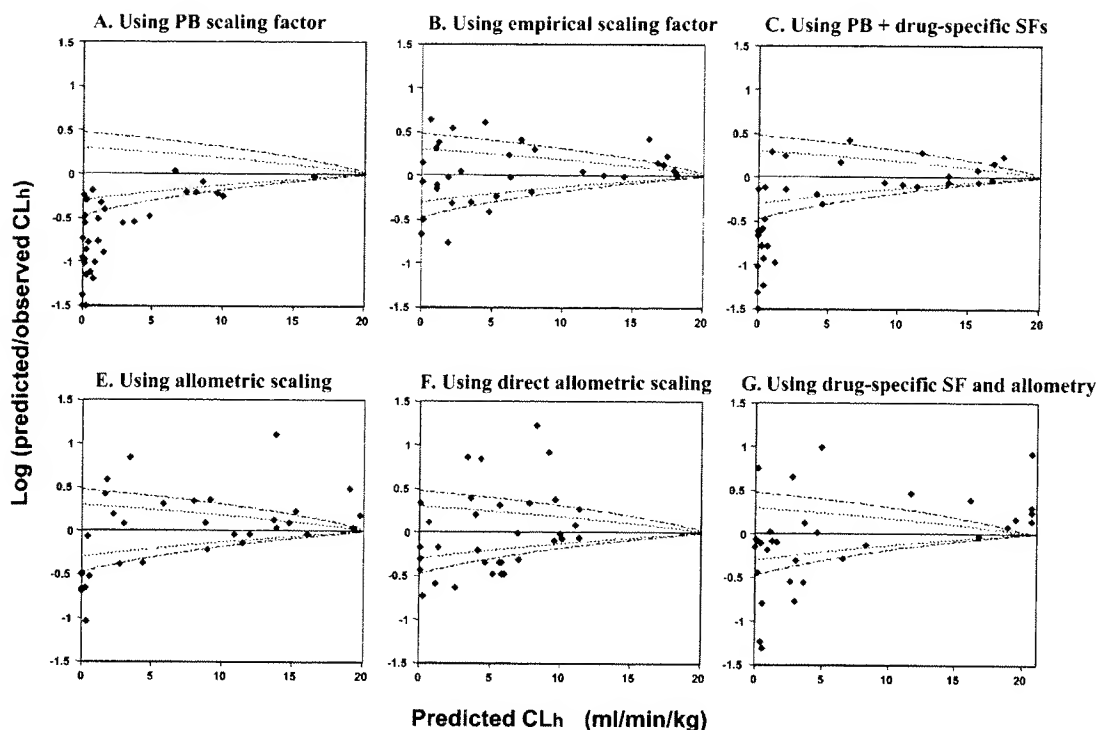


Fig. 4. Precision error (expressed as the log of the predicted/observed CL_h ratio) for predicted CL_h for 33 drugs using six different approaches. (A) Using PB-SF, (B) using empirical SF, (C) using PB-SF and drug-specific SF, (E) using allometric scaling, (F) direct allometric scaling, and (G) using drug-specific SF and allometry. The light and heavy dotted lines indicate the limits for the CL_h prediction when either a 2- or 3-fold error, respectively, on the CL_{int} prediction propagated into CL_h .

ing site in both matrices to illustrate the potential difference between plasma and microsomes. Plotting the unbound fraction in microsomes (at the protein concentration of 1 mg/ml) against the unbound fraction in plasma (40 g/ml) indicates that only in the case of neutral drugs are the affinity constants comparable. However $f_{u,m}$ differs from $f_{u,p}$ because of difference in protein concentration. In order to achieve a similar fraction unbound in microsomes (commonly used protein concentration of 1 mg/ml) and plasma the microsomal K_a

should exceed plasma by 40-fold. Using nonlinear regression a clear difference between the affinity constants in the two matrices can be quantified for bases ($K_{a,m} > K_{a,p}$ by 8.7) and for acids ($K_{a,p} > K_{a,m}$ by 45). The substantial difference in K_a observed for bases probably reflects the extensive binding of these drugs to lipid whereas for acids the nature and concentration of protein dominates. For the former class of drugs, $f_{u,m}$ will coincide with $f_{u,p}$ if a microsomal suspension of approximately 5 mg/ml protein concentration is used.

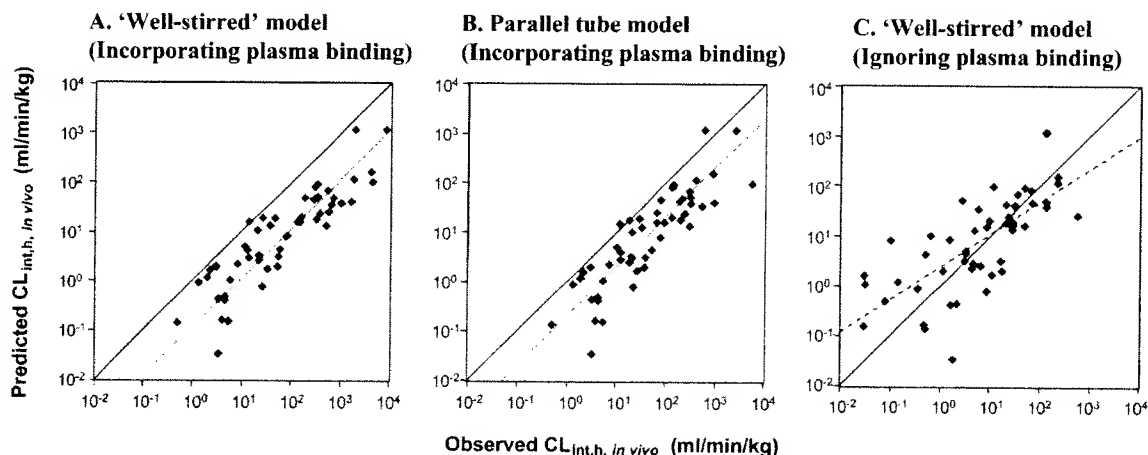


Fig. 5. Correlation between the observed and predicted human CL_{int} for a dataset of 52 drugs using the PB scaling factor based on microsomal recovery and two different liver models [well-stirred (panels A and C) and parallel tube (panel B) models]. Plasma protein binding was incorporated in panels A and B and ignored in panel C. Lines represent the regression with a fixed (A, B) or non-fixed (C) slope (dotted) and unity (solid).

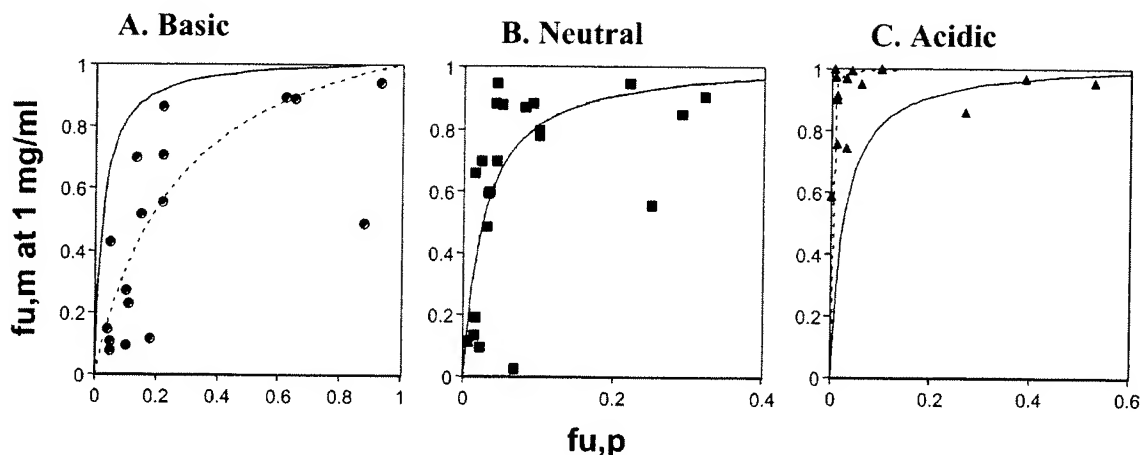


Fig. 6. Relationship between nonspecific binding of basic (panel A), neutral (panel B), and acidic (panel C) drugs in microsomes and plasma. The $f_{u,m}$ values refer to a protein concentration of 1 mg/ml and $f_{u,p}$ to 40 mg/ml. Solid lines represent the simulated lines based on Eq. 18 assuming the same affinity constant in plasma and microsomes, and dotted lines represent the simulations using microsome/plasma affinity constant ratios of 8.7 (panel A), 1.02 (panel B), and 0.022 (panel C) obtained by nonlinear regression.

The use of an empirical SF (method B) to overcome the bias in prediction increases value of the scaling factor over the PB value determined from microsomal recovery (0.86 g protein/kg) to a value of 7.9 g protein/kg. While this provides an immediate, pragmatic solution for systematic underprediction, in the longer term it is important to identify explanations and modify scaling strategies to account for these confounding issues. Previous analyses of rat predictions from both hepatocytes and hepatic microsomes did not show a systematic bias (2,34). This undoubtedly reflects the genetic and environmental constancy that operates with animal experimentation. Thus the drug metabolizing enzymes isolated and monitored *in vitro* are essentially identical to those operating *in vivo*. For human tissue experimentation there is an impact from extrinsic factors such as tissue handling and storage procedures (often not detailed) that may increase variability *in vitro* beyond that evident *in vivo*. Substantial inter-individual variability in drug clearance is well known and the mismatch between the liver donors and the young healthy volunteers used for most *in vivo* studies are important. The systematic underprediction of human CL_{int} from *in vitro* human hepatic microsomal studies may be, at least in part, a reflection of this situation.

A number of factors are of more importance for human than rat clearance studies. One is the role of CYP3A enzymes, the most abundant and most used subfamily of human enzymes. Their atypical kinetics and propensity to activation phenomena are important considerations for clearance predictions (37). Also the expression of these enzymes extrahepatically may have a major impact (38–40). Until the relative importance of the above factors, amongst others, to the overall underprediction phenomenon is established a pragmatic approach of adopting an empirical SF is recommended.

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EXHIBIT

18



Integrated oral bioavailability projection using in vitro screening data as a selection tool in drug discovery

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Abstract

The objective of the analysis described herein is to examine the in vitro/in vivo relationship of estimated bioavailability values and also the applicability of the estimated in vitro bioavailability to lead candidate selection in drug discovery. To this end, in vitro ADME data from screening assays as well as in vivo rat pharmacokinetic (PK) data were compiled for 140 compounds across therapeutic areas from the Pfizer library in Ann Arbor. The compounds span a broad range of structural types, including neutral, basic, and acidic compounds. Solubility and Caco-2 permeability data from in vitro ADME screening were used to calculate the fraction dose absorbed (FDP) using the physiologically based IDEA[®] model. In vitro metabolic stability ($t_{1/2}$) from human and rat liver microsomal incubations was converted to an in vitro intrinsic clearance value (CL'_{int}), which was then scaled up to reflect in vivo clearance (CL) and hepatic extraction as described by Obach et al. [J. Pharmacol. Exp. Ther. 283 (1997) 46]. Subsequently, the in vitro/in vivo relationship between the measured bioavailability (F_{obs}) in rats and the estimated bioavailability (F_{est}) from FDP and predicted CL values was examined. The observed data suggest that compounds with low estimated in vitro bioavailability ($F_{est} < 15\%$) are more likely to have low in vivo bioavailability ($F_{obs} < 30\%$). Therefore, the present study indicates that in vitro estimation of bioavailability is an efficient tool to eliminate compounds having low bioavailability prior to in vivo characterization and therefore can be used to reduce attrition due to poor ADME properties in drug development.

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Keywords: IDEA model; Bioavailability; In vitro prediction; Drug discovery

1. Introduction

A major challenge to pharmaceutical scientists in drug discovery is in the optimization and selection of lead compounds from the abundance of new chemical entities (NCEs) in early drug discovery with the best chances for success. Many studies have supported that

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poor pharmacokinetic properties heavily contribute to compound failure or at a minimum, difficulty of the compound to progress through drug development (Prentis et al., 1998; Eddershaw et al., 2000). Therefore, to reduce attrition in drug development, it is important to identify the major pharmacokinetic hurdles of drug candidates and assess the developability of a compound early in drug discovery. Recently, in vitro ADME screening assays have been established to generate significant amounts of in vitro ADME data (e.g. solubility, permeability, metabolic stability, drug–drug interaction potential, etc.) in a high throughput format early in drug discovery. The availability of such in vitro ADME data enables their use to characterize and predict in vivo pharmacokinetics of drugs (Venkatesh and Lipper, 2000; Hodgson, 2001). Consequently, there is growing interest to establish the in vitro/in vivo correlation of pharmacokinetic parameters in order to select lead candidates having favorable pharmacokinetic properties without any significant in vivo evaluation.

Many studies have been published exploring different approaches to establish in vitro/in vivo correlations by combining metabolic clearance and fraction dose absorbed to ultimately project human pharmacokinetic parameters (Obach et al., 1997; Obach, 1999; Shibata et al., 2000; Lave et al., 1997; Naritomi et al., 2001; Chiou and Barve, 1998; Chiou et al., 2000). However, there have been limited attempts to integrate multiple in vitro ADME parameters from high throughput screening to estimate bioavailability of NCEs and subsequently to use the estimated in vitro bioavailability as a screening filter in the early stages of drug discovery (Theil et al., 2003; Parrot and Lavé, 2002; Bohets et al., 2001; Waterbeemd et al., 2001; Waterbeemd, 2001). Since bioavailability is affected by multiple factors, including solubility, permeability, and first pass metabolism, in vitro biopharmaceutical/pharmacokinetic parameters from screening assays should not be used in isolation to project oral bioavailability unless the critical factor limiting bioavailability has been clearly identified for the particular compound or chemical series. Therefore, if multiple in vitro screening measurements can be integrated to adequately estimate bioavailability, it will greatly reduce the cost and time consuming in vivo pharmacokinetic evaluation of discovery compounds. In order for an integrated model to gain acceptance by discovery

project teams (relative to discrete models), it must clearly demonstrate the ability to choose a greater proportion of ‘good’ compounds with a minimized risk for eliminating otherwise ‘good’ compounds from the discovery project. The latter compounds are termed as ‘false negatives’ and are a general cause for concern in early discovery. Additionally, such an integrated model should be broadly applicable to early discovery programs across structural series and therapeutic areas.

The present study examines the in vitro/in vivo relationship of estimated bioavailability values and also the applicability of the predicted bioavailability to the lead candidate selection in early drug discovery. The methodology used in this study was to combine in vitro data with a commercially available software package (IDEA[®], LION Bioscience Inc., San Diego, CA, USA) with the goal of implementing this tool in early drug discovery. In vitro ADME data from screening assays as well as in vivo rat PK data were compiled for 140 compounds across a broad range of structural chemotypes from the Pfizer Chemical library in Ann Arbor. A retrospective analysis of in vitro/in vivo relationship was performed between the measured bioavailability in rats and the in vitro bioavailability estimated from integration of in vitro screening data.

2. Methods

2.1. Fraction dose absorbed (FDp)

Fraction dose absorbed in portal vein (FDp) was estimated by using the IDEA[®] model (LION Bioscience Inc., 92121) (Theil et al., 2003; Parrot and Lavé, 2002). As shown in Fig. 1, the input factors used in IDEA[®] model to estimate FDp values are solubility at various pH values (1.5, 5.0, 6.5, 7.0, and 7.5), Caco-2 permeability ($P_{app(A \rightarrow B)}$) measured at 20 μ M, log P and the efflux ratio ($P_{app(B \rightarrow A)}/P_{app(A \rightarrow B)}$). Experimental procedures to determine Caco-2 permeability, solubility, and log P were discussed previously (Stilgenbauer et al., 2000a,b; Kibbey et al., 2001). Solubility at various pH values was calculated based on measured solubility at pH 6.5 and pK_a from in vitro ADME screening as described by Flynn et al. (Kramer and Flynn, 1972; Horter and Dressman, 1997).

Fig. 1. In vitro data input to an IDEA[®] model.

2.2. Clearance (CL)

In vivo clearance was projected based on in vitro disappearance half-life measured from rat and human liver microsomal incubation by using the following equations, as summarized by Obach et al. in their previous report (Obach, 1999).

$$CL'_{\text{int}} = 0.693 \times \frac{1}{t_{1/2} \text{ (min)}} \times \frac{\text{gm liver wt}}{\text{kg body wt}} \times \frac{\text{ml incubation}}{\text{mg microsomal prot}} \times \frac{45 \text{ mg microsomal prt}}{\text{gm liver wt}} \quad (1)$$

(CL'_{int} : hepatic intrinsic clearance)

$$CL = \frac{Q \times CL'_{\text{int}}}{Q + CL'_{\text{int}}} \quad (Q : \text{hepatic blood flow}) \quad (2)$$

The use of in vitro hepatic microsomal intrinsic CL data to predict systemic CL was based on several assumptions: (1) metabolic CL is the primary CL mechanism of compounds, (2) the liver

is the major CL organ, (3) oxidative microsomal metabolism is the predominant route of metabolism (compared with non-microsomal metabolism and conjugative metabolism), and (4) metabolic rates and enzyme activities measured in vitro are truly reflective of those that occur in intact systems in vivo.

Experimental procedures to determine in vitro disappearance half-life in microsomes were discussed previously (Stilgenbauer et al., 2000a,b).

2.3. Bioavailability (F, %)

Oral bioavailability can be determined using the following equations.

$$F = F_a(1 - E_h) = F_a \left(1 - \frac{CL_h}{Q}\right) \quad (3)$$

where F_a is fraction dose absorbed; E_h is hepatic extraction ratio; CL_h is hepatic blood clearance, and Q is hepatic blood flow (Rowland and Tozer, 1995).

Assuming that hepatic metabolism is the primary clearance mechanism of compounds, $CL_h = CL$

where CL is systemic clearance, thus

$$F = F_a \left(1 - \frac{CL}{Q} \right) \quad (4)$$

In the present study, in vitro projected oral bioavailability (F_{est}) was estimated based on FD_p (projected F_a by using computational IDEA model: see details in Section 2) and projected in vivo clearance (CL) (see Eqs. (1) and (2)). Therefore,

$$F_{\text{est}} = FD_p \left(1 - \frac{CL}{Q} \right) \quad (5)$$

where $Q = 70$ ml/min/kg in rats and 21 ml/min/kg in humans (Altman and Dittmer, 1972).

2.4. Statistical analysis

For each method, a test was performed to assess the association between each method's classification and the in vivo classification. The null hypothesis for each test was no association and the continuity-adjusted c^2 statistic (c^2_{adj}) was used as the statistic for the test.

3. Results

3.1. In vitro profiles of compounds

In vitro profiles of structurally diverse 140 compounds are illustrated in Fig. 2. Binning which is a classification scheme to categorize numerical data into various segments (e.g. high, medium, and low) allows for a simple assessment of the property's distribution and also to determine where a potential liability exists. For example, it is clear that the majority of the compounds have poor aqueous solubility (86/140) or poor Caco-2 permeability (65/140) suggesting that poor absorption is likely to be a contributing factor for poor in vivo performance for these compounds. The choice of 10 $\mu\text{g/ml}$ as a minimum acceptable solubility is based on Lipinski's analysis where for a compound with high permeability and a dose of 1 mg/kg, aqueous solubility must be equal or greater than 10 $\mu\text{g/ml}$ for complete absorption in humans (Lipinski, 2000). The low permeability cut-off was established based on in-house data for the Biopharmaceutical Classification Scheme (BCS) compounds in our Caco-2 assay.

3.2. Classification of drugs by using in vitro and in vivo bioavailability

Since the methodology applied to predict in vivo clearance does not include non-microsomal routes of elimination nor non-metabolic elimination pathway (see Section 2), a linear relationship between in vitro and in vivo estimates of bioavailability would not be anticipated. Indeed, there is no linear correlation between in vitro and in vivo estimates of bioavailability (discussed in details later). However, the observed data suggest that compounds with low estimated in vitro bioavailability (F_{est}) are more likely to have low in vivo bioavailability in rats ($F_{\text{obs}} < 30\%$). Therefore, another aspect of the in vitro/in vivo relationship was examined based on *drug classification* by using both F_{est} and F_{obs} .

3.2.1. Set the optimal boundary for the classification of compounds

It is well recognized that compounds with low bioavailability tend to have higher clinical variability as demonstrated by Hellriegel et al. (1996). Based on the report from Hellriegel et al. in conjunction with our internal experience, in vivo bioavailability of 30% was used as the minimum threshold of in vivo bioavailability (F_{obs}) to assess the developability of compounds. To find an optimum range of F_{est} (%) for classifying low orally available compounds ($F_{\text{obs}} < 30\%$), misclassification rates were examined as shown in Fig. 3. The ideal region for classifying compounds would simultaneously minimize both false negative and false positive rates. Fig. 3 suggests that classifying observations with F_{est} less than 15% as low bioavailability provides an optimal balance between both false negative and false positive rates.

3.2.2. Binning relationship between F_{obs} and F_{est}

As shown in Fig. 4, there is good correlation between in vitro estimates of bioavailability (F_{est}) for rats and humans. However, 12 compounds out of 140 compounds were classified differently by using in vitro estimates of bioavailability (F_{est}) in humans or rats. For example, some of compounds (open circles in Fig. 4) showed greater than 15% of F_{est} in rats but less than 15% of F_{est} in humans. Therefore, to minimize false negatives, greater than 15% of F_{est} in either

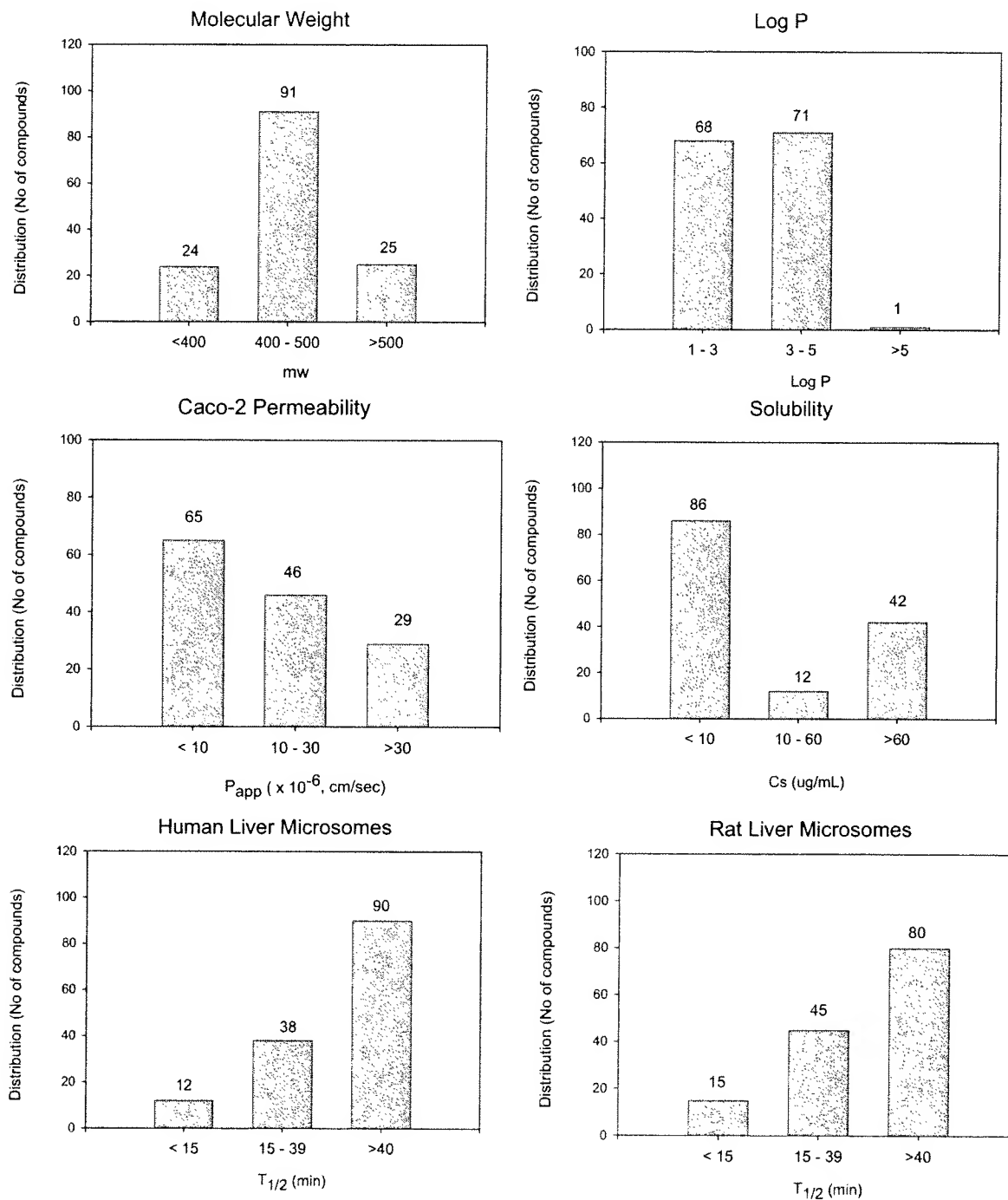


Fig. 2. Distribution of compounds properties in the data set: human liver microsomal $t_{1/2}$ (min) < 15, 15–39, and ≥ 40 is equivalent to $CL'_{int} > 87$, $CL'_{int} = 34$ –87, and $CL'_{int} < 34$ ml/min/kg, respectively. Rat liver microsomal $t_{1/2}$ (min) < 15, 15–39, and ≥ 40 is equivalent to $CL'_{int} > 166$, $CL'_{int} = 64$ –166, and $CL'_{int} < 64$ ml/min/kg, respectively.

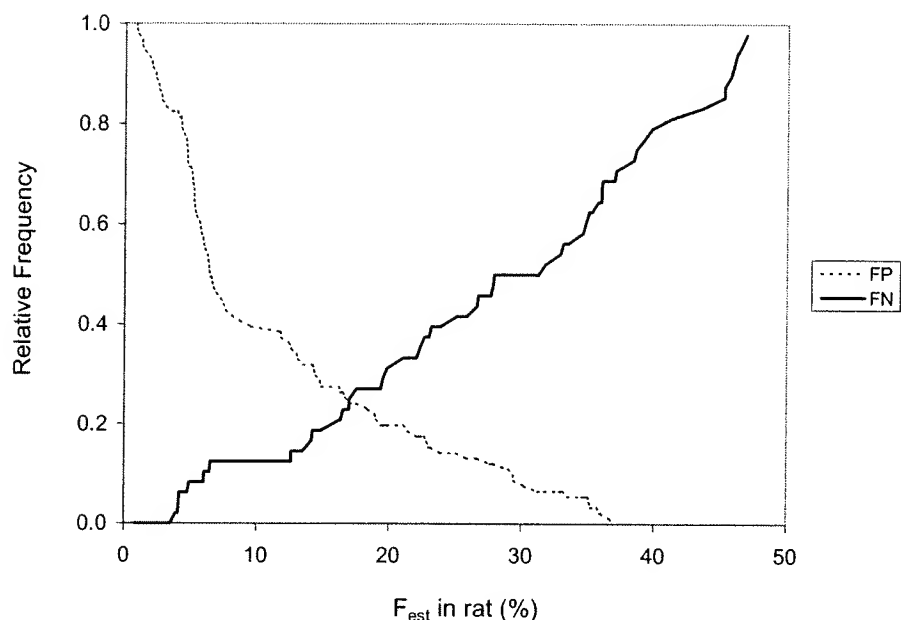


Fig. 3. In vitro estimated bioavailability in rat versus relative frequency of false negative and false positive (misclassification rates): FP: false positive, FN: false negative.

rat or humans was used for the compound selection to achieve greater than 30% bioavailability in vivo. As illustrated in Fig. 5, among 140 compounds, 55 compounds were classified as the Category I (less than 15% of F_{est} and less than 30% of F_{obs}), 9 compounds into Category II (false negative), 47 compounds into

Category III (greater than 15% of F_{est} and greater than 30% of F_{obs}), and 29 compounds into Category IV (false positive).

Collectively, 73% of compounds were classified into the correct category using this approach without significant false negatives (6%). Statistical analysis

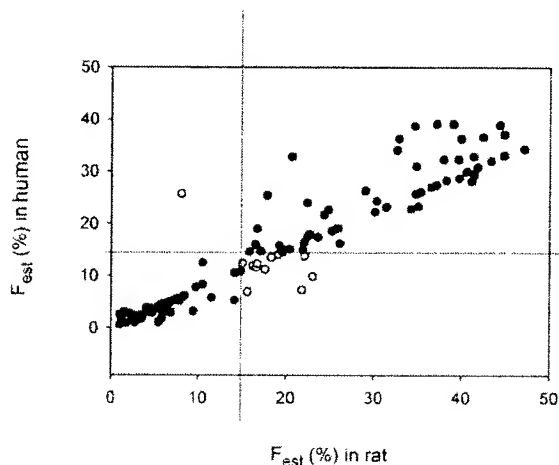


Fig. 4. In vitro estimated bioavailability in humans vs. in vitro estimated bioavailability in rats: open circles represent the compounds that were classified differently in humans and rats.

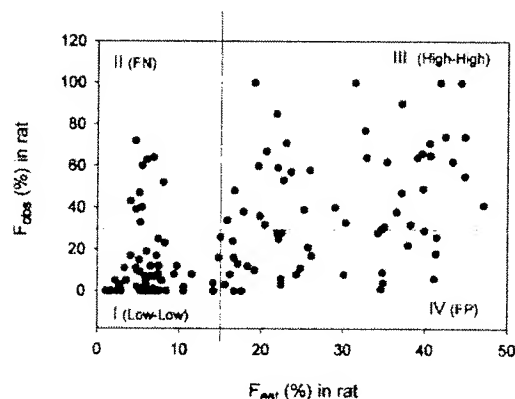


Fig. 5. Binning relationship between in vitro/in vivo bioavailability. Each category represents as follows; Category I: less than 15% of F_{est} and less than 30% of F_{obs} , Category II: FN, false negative, Category III: greater than 15% of F_{est} and greater than 30% of F_{obs} , and Category IV: FP, false positive.

Table 1
Comparison between discrete analysis (single parameter analysis) and integrated in vitro bioavailability projection method for the classification of compounds

In vitro	In vivo (Rat)	
	Bad ($F_{\text{obs}} < 30\%$)	Good ($F_{\text{obs}} > 30\%$)
(A) Integrated in vitro bioavailability projection method		
Bad ($F_{\text{est}} < 15\%$)	55 (39.3%)	9 (6.4%)
Good ($F_{\text{est}} > 15\%$)	29 (20.7%)	47 (33.6%)
(B) Discrete analysis		
Bad ($C_s < 10$ or $\text{RLM} < 15$ or $P_{\text{app}} < 10$)	73 (52.1%)	24 (17.1%)
Good ($C_s > 10$, $\text{RLM} > 15$ and $P_{\text{app}} > 10$)	11 (7.9%)	32 (22.9%)

C_s = solubility ($\mu\text{g/ml}$), $\text{RLM} = t_{1/2}$ in rat microsome (min) and P_{app} = Caco-2 permeability ($\times 10^{-6}$, cm/s). $\text{RLM} < 15$ is equivalent to $\text{CL}'_{\text{int}} > 166 \text{ ml/min/kg}$.

(adjusted chi-square test) indicated that there is a significant association (P -value < 0.0001) between this in vitro and in vivo classification method. Furthermore, the results were compared with the discrete analysis that utilizes each in vitro parameter separately to classify compounds. As summarized in Table 1, the discrete analysis classified 75% of compounds into the correct category but it generated a threefold higher rate of false negatives (17%) than the integrated in vitro projection method (6%). Additionally, the integrated model discriminated 'good' compounds from the 'bad' compounds at a higher rate than the discrete model (22.9% discrete versus 33.6% integrated models). Thus, the observed data suggest that integrated in vitro projection of bioavailability (F_{est}) can be an efficient tool to screen out compounds having low bioavailability prior to in vivo characterization in early discovery.

4. Discussion

Early assessment of ADME profiles of compounds is important in reducing attrition in drug discovery, and consequently expediting the drug development process. The major pharmacokinetic parameters taken into account at lead candidate selection stages are systemic exposure (AUC), bioavailability (F), and plasma half-life ($t_{1/2}$). Systemic exposure (AUC) is assessed to determine whether systemic exposure associated

with efficacy can be achieved with clinically applicable doses, and thus the systemic exposure should be considered in the conjunction of potency/efficacy of the drugs. Since the desired systemic exposure of drug candidates depends on the pharmacological activity against targets, the first cut-off of AUC as selection criteria of lead candidates will be target specific or project specific. Plasma half-life is also an important factor to support twice a day dosing (b.i.d.) or once a day dosing (q.d.) regimen and it is altered by the change of other pharmacokinetic parameters, such as clearance and volume of distribution. In vitro ADME screening data provide useful information regarding pharmacokinetic parameters deemed to be important in early discovery. Therefore, for the optimization of plasma half-life, there have been many different approaches to predict those factors responsible for affecting in vivo plasma half-life from in vitro ADME data (Obach et al., 1997; Obach, 1999; Shibata et al., 2000; Lave et al., 1997; Naritomi et al., 2001; Chiou and Barve, 1998; Chiou et al., 2000; Poulin and Theil, 2002).

Oral bioavailability is a particularly important selection criterion for lead candidates in early drug discovery, considering that oral administration is the most desirable route of administration. Therefore, there have been different approaches to predict bioavailability (ultimately for humans) (Theil et al., 2003; Parrot and Lavé, 2002; Bohets et al., 2001; Waterbeemd et al., 2001; Waterbeemd, 2001) but still it is needed to further refine those approaches for more accurate prediction of human bioavailability. In order to meet the throughput demand of discovery, and to identify lead compounds in a timely manner, a practically facile approach is more appropriate to predict bioavailability of compounds in early drug discovery while a more refined approach is required for compounds in advanced stages of drug development. Therefore, the present study is focused on the utility of in vitro screening data in predicting bioavailability of early discovery compounds. As shown in Fig. 2, binning 1 property allows for a simple assessment of the compound's property distribution and to determine where a potential cause of low bioavailability exists. However, since bioavailability is determined by combining multiple factors including solubility, permeability and metabolic stability, in vitro ADME data from screening assays should not be used in isolation to project oral bioavailabil-

ity unless the critical factor limiting bioavailability has been clearly identified for particular compounds or chemical series. Indeed, the discrete analysis that utilizes each in vitro ADME data separately produced a significant rate of false negatives (17%) compared to the integrated model (6%) further highlighting this caveat (Table 1).

A set of 140 structurally diverse compounds was selected across therapeutic areas, and the relationship between in vitro and in vivo estimates of bioavailability was examined. The observed data suggest that compounds with low estimated in vitro bioavailability (F_{est}) are more likely to have low in vivo bioavailability ($F_{\text{obs}} < 30\%$). As shown in Fig. 5, using 0–15% of F_{est} as an indicator of compounds with low in vivo bioavailability ($F_{\text{obs}} < 30\%$) could classify 73% of the compounds into the correct category, with 6% classified as false negatives and 21% classified as false positives. On the other hand, although the discrete analysis classified 75% of compounds into correct category it chose, only 22.9% of compounds that ultimately demonstrated adequate in vivo bioavailability ($>30\%$) compared to 33.6% by the integrated model. While the integrated method does not offer a superiority when solely considering elimination as the goal (40% versus 52%), the combination of higher true positives and low false negatives of the integrated model supports the integrated method as a complementary tool to the discrete analysis for practitioners of early ADME screening in support of various drug discovery programs.

As with any model, our analysis of this integrated approach yields both false positives and false negatives. The observation of false positives can be explained in several ways. First, the present approach utilizes in vitro hepatic microsomal intrinsic CL data to predict systemic CL. Therefore, if the compounds do not meet the assumptions described in Section 2, errors in both CL and bioavailability projections will occur. For example, if Phase II reactions (conjugation) are the major elimination route for the compounds, the present approach using microsomal stability data will underestimate hepatic clearance of those compounds and subsequently in vitro estimates of bioavailability (F_{est}) will be greater than in vivo bioavailability (false positive). Second, the IDEA[®] model does not incorporate a GI stability factor into the calculation of fraction dose absorbed, and thus if compounds undergo signif-

icantly intestinal metabolism, or chemical instability in gastric acid, then the bioavailability of those compounds will be overestimated by the present approach using calculated FD_p values from IDEA[®] model.

While false positive data are of less concern in the present approach, false negative data should be more carefully evaluated in order to reduce the chance of eliminating promising compounds. In the present study, only 6% of tested compounds were classified as the false negatives. The specific reasons for these failures were not examined further in this study. However, based on evidence from literature and our experience, several reasons could contribute to these observations. First, if active transport mechanism is involved in the intestinal drug absorption, Caco-2 data may underestimate the intestinal absorption of those compounds due to lower expression of active uptake transporters relative to in vivo expression, and subsequently underestimate the fraction dose absorbed. Consequently, in vitro estimates of bioavailability using underestimated fraction dose absorption should be less than measured values in vivo. Second, if there is significant difference between in vitro and in vivo solubility (e.g. increased solubilization by bile salts in vivo, etc.), the IDEA[®] model may underestimate the fraction dose absorption of poorly soluble drugs and subsequently underestimate the in vivo bioavailability. Third, if the metabolic rates and enzyme activities measured in vitro are significantly different from those that occur in vivo, the present approach using in vitro metabolic stability data may overestimate the in vivo clearance and subsequently may result in the underestimation of in vivo bioavailability.

While there is a need for further clarification of false negatives, this does not diminish the utility of our approach for the following reasons: (a) the risk was superior to the risk associated with decision making based on discrete variables (6% versus 17% false negative); (b) there was no discernible trend in the false negatives based on the scaffold or template suggesting a systematic deviation; and (c) the risk associated with 6% of false negative is generally deemed as acceptable at the early stages in drug discovery.

In conclusion, the results suggest that the present approach using in vitro estimate of bioavailability is useful (1) to reduce the time and cost of in vivo animal studies and (2) eliminate compounds having low bioavailability prior to in vivo characterization. This

study also underscores the need to integrate various in vitro ADME data in a scientifically sound and practically facile manner in order to meet the throughput and data turnaround times in early drug discovery.

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EXHIBIT

19

PREDICTION OF HUMAN HEPATIC CLEARANCE FROM IN VIVO ANIMAL EXPERIMENTS AND IN VITRO METABOLIC STUDIES WITH LIVER MICROSOMES FROM ANIMALS AND HUMANS

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ABSTRACT:

We investigated the quantitative prediction of human hepatic metabolic clearance from in vitro experiments focusing on cytochrome P450 metabolism with eight model compounds, FK1052, FK480, zolpidem, omeprazole, nicardipine, nilvadipine, diazepam, and diltiazem. For the compounds, in vivo human hepatic extraction ratios ranged widely from 0.03 to 0.87. In vitro and in vivo hepatic intrinsic clearance (CL_{int}) values for each compound were measured and calculated in rats and/or dogs and humans. $CL_{int, in vitro}$ was determined from a substrate disappearance rate at 1 μ M in hepatic microsomes, which was a useful method. $CL_{int, in vivo}$ was calculated from in vivo pharmacokinetic data using three frequent mathematical models (the well stirred, parallel-tube, and dispersion models). The human scaling factor values ($CL_{int, in vivo}/CL_{int, in vitro}$) showed marked difference

among the model compounds (0.3–26.6-fold). On the other hand, most of the animal scaling factors were within 2-fold of the values in humans, suggesting that scaling factor values were similar in the different animal species. When human $CL_{int, in vitro}$ values were compared with the actual $CL_{int, in vivo}$, correlation was not necessarily good. By contrast, using human $CL_{int, in vitro}$ corrected with the rat and/or dog scaling factors yielded better predictions of $CL_{int, in vivo}$ that were mostly within 2-fold of the actual values. Furthermore, successful predictions of human CL_{oral} and hepatic extraction ratio (E_H) were obtained by use of the human $CL_{int, in vitro}$ corrected with animal scaling factors. The new variant method is a simple one, incorporating additional information from animal studies and providing a more reliable prediction of human hepatic clearance.

In recent years, the process of drug discovery and development has become an increasingly time-consuming and costly endeavor. Much of the time and cost are expended on generating data that support the efficacy and safety profiles of the drug. Safe and efficient drug candidates must therefore be selected before clinical trials.

On the other hand, there is growing awareness of the key roles that pharmacokinetics and drug metabolism play as determinants of in vivo action. In these situations, early pharmacokinetic investigation plays an increasingly important role in the optimization and selection of drug candidates. In particular, it is important to predict human hepatic metabolic clearance because many drugs are eliminated from the body by hepatic metabolism. For predicting hepatic clearance, theoretical aspects of in vitro/in vivo scaling, based on a physiological

model and clearance concepts, have been developed (Rane et al., 1977; Lin et al., 1982; Roberts and Rowland, 1986; Wilkinson, 1987). Application of this method has been successful in predicting in vivo hepatic clearance in rats for many drugs metabolized by P450¹ from in vitro metabolism data using rat liver microsomes and isolated hepatocytes (Sugiyama et al., 1988; Houston, 1994). Since human liver samples have become more readily available, it would also be very useful to predict in vivo from in vitro data in humans. However, there has been relatively limited application of this approach (Hoener, 1994; Iwatsubo et al., 1997b), and there have been many failed attempts at predicting human hepatic clearance. For example, Iwatsubo et al. (1997a) reported a comparison of $CL_{int, in vitro}$ and $CL_{int, in vivo}$ for 25 metabolic reactions in humans from literature data. According to the report, although $CL_{int, in vitro}$ generally exhibited a positive correlation with $CL_{int, in vivo}$, more than a 3-fold difference was observed in CL_{int} for about 50% of the 25 metabolic reactions. Houston and Carlile (1997) also compared $CL_{int, in vitro}$ obtained from in vitro experiments using rat liver microsomes with $CL_{int, in vivo}$ for 28 drugs metabolized by P450. Although the predictability of $CL_{int, in vivo}$ from in vitro data was good overall, the results of some of the drugs tended to be low estimates. To improve the predictions of human hepatic clearance, a few investigators have described new methods and approaches. For example, Lave et al. (1997) have proposed the allometric scaling techniques combined with in vitro data. Obach (1999) has

¹ Abbreviations used are: P450, cytochrome P450; HPLC, high-performance liquid chromatography; CL, plasma clearance; CL_H , hepatic clearance; CL_{int} , intrinsic metabolic or hepatic clearance; CL_R , renal clearance; D_N , dispersion number; E_H , hepatic extraction ratio; F_a , the fraction absorbed from the intestinal tract; F_H , hepatic availability; f_p , unbound fraction in plasma (or serum); $f_{u, microsome}$, unbound fraction in microsome; Q_H , hepatic blood flow rate; R_B , blood-to-plasma concentration ratio.

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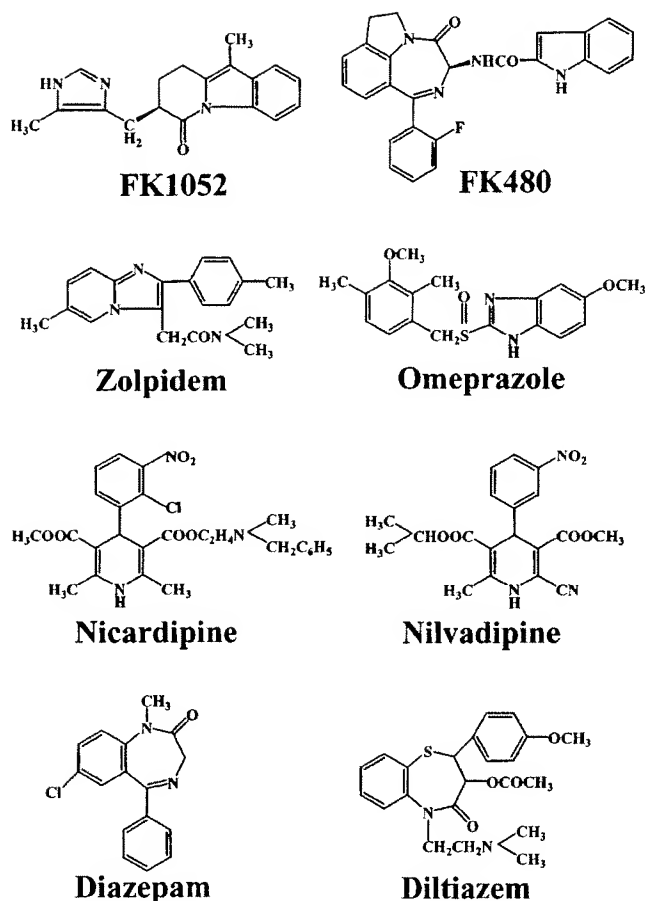


FIG. 1. Chemical structures of model compounds.

reported that inclusion of microsome binding values in the prediction of clearance from *in vitro* data appears to be a more broadly applicable approach.

In the present study, we have examined *in vitro* and *in vivo* metabolic clearance of eight model compounds, FK1052, FK480, zolpidem, omeprazole, nicardipine, nilvadipine, diazepam, and diltiazem, and calculated the $CL_{int, in vitro}$ and $CL_{int, in vivo}$ using *in vitro* and *in vivo* metabolism data in rats, dogs, and humans. At the same time, the measurement method of $CL_{int, in vitro}$, which is determined from a substrate disappearance rate at 1 μ M in hepatic microsomes, was used as a simple and useful method. We have also compared the parameters and evaluated a quantitative prediction method of human hepatic clearance focusing on P450 in drug discovery.

Materials and Methods

Chemicals. FK1052, FK480, and nilvadipine were synthesized by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Zolpidem hemitartrate and omeprazole sodium were kindly provided by Fujisawa-Synthelabo Pharmaceuticals (Tokyo, Japan) and Astra Japan, Ltd. (Osaka, Japan), respectively. Diltiazem hydrochloride and nicardipine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Diazepam was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NADP⁺, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. The other reagents and solvents used were of analytical and HPLC grade.

Selection of Model Compounds. FK1052, FK480, omeprazole, zolpidem, nicardipine, nilvadipine, diltiazem, and diazepam (Fig. 1) were selected as the model compounds based on the following conditions:

- Clearances of model compounds are determined by hepatic P450 metabolism
- Extrahepatic clearances are assumed to be negligible
- *In vivo* pharmacokinetic parameters in rats and/or dogs, and humans are reported
- Absorption rates are good with no species difference

Although it has been reported that diltiazem is metabolized in part by liver microsomal esterase in rats (LeBoeuf and Grech-Bélanger, 1987), the compound was examined for reference.

Hepatic Microsomes. Liver specimens from adult male Sprague-Dawley rats (250–270 g, $n = 3$; Charles River Japan, Inc., Yokohama, Japan) and adult male dogs (9.5–10 kg, $n = 3$; Japan Laboratory Animals, Inc., Tokyo, Japan) were rinsed and homogenized with ice-cold 1.15% KCl. These pooled microsomes were prepared by differential centrifugation, and the 105,000g pellet was rinsed and resuspended in 1.15% KCl. Pooled human microsomes were obtained from Human Biologics International (Scottsdale, AZ). The pooled human microsomes were prepared from 15 individual liver donors that were selected on the basis of having average activities for the major P450 isozymes (1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, and 4A11). Each suspension was divided into aliquots, frozen, and stored at -80°C until used.

***In Vitro* Metabolism in Microsomes.** *In vitro* experiments. The time courses of the unchanged model compounds in microsomes were obtained. Each compound was incubated with a reaction mixture (500 μ l) consisting of animal or human liver microsomal protein and NADPH-generating system (2 mM NADP⁺, 10 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 5 mM MgCl₂) in the presence of 100 mM potassium phosphate buffer (pH 7.4). After preincubation at 37°C for 5 min, enzyme reactions were initiated by adding 5 μ l of model compound solution in methanol. The final concentration of each model compound used was 1 μ M. The microsomal concentrations used were 0.2 mg/ml (nicardipine, nilvadipine, and diltiazem), 0.5 mg/ml (FK1052, zolpidem, omeprazole, and diazepam), and 1.0 mg/ml (FK480). After incubation at 37°C for various time periods, the reactions of FK1052, FK480, omeprazole, zolpidem, nicardipine, and diltiazem were terminated by the addition of 500 μ l of acetonitrile. The reactions of diazepam and nilvadipine were terminated by adding 500 μ l of methanol and 3 ml of ethyl acetate, respectively. After stopping the metabolic reactions, the reaction mixtures of FK1052, FK480, omeprazole, zolpidem, nicardipine, diltiazem, and diazepam were centrifuged at 10,000g for 5 min, and an aliquot of the supernatant was injected on an HPLC for measuring the unchanged compound concentration. The reactions of nilvadipine were processed by extraction. The organic fraction was evaporated under N₂, and the residue was reconstituted in the mobile phase (see below) for HPLC analysis.

Determination of unchanged model compound concentrations. An LC module I plus (Millipore Co., Milford, MA) was used. The column for the analysis was an Inertsil ODS-3 (5 μ m, 150 \times 4.6 mm) (GL Science, Inc., Tokyo, Japan). The flow rate was 1.0 ml/min. The mobile phase and detection wavelength for the analysis of each model compound was as follows: FK1052, mobile phase: buffer A (5 mM phosphate buffer, pH 7.2)/CH₃CN (50:50), detection: UV 242 nm; FK480, mobile phase: buffer A/CH₃CN (40:60), detection: UV 295 nm; Zolpidem, mobile phase: buffer A/CH₃CN (55:45), detection: UV 254 nm; Omeprazole, mobile phase: buffer A/CH₃CN (60:40), detection: UV 302 nm; Nicardipine, mobile phase: buffer A/CH₃CN (33:67), detection: UV 240 nm; Nilvadipine, mobile phase: buffer A/CH₃CN (40:60), detection: UV 245 nm; Diazepam, mobile phase: buffer A/CH₃OH (35:65), detection: UV 254 nm; Diltiazem, mobile phase: buffer A/CH₃CN (50:50), detection: UV 240 nm.

All assay methods showed the concentration range of 0.1 to 2 μ M. Reproducibility was evaluated by performing five replicate analyses of microsomal samples containing 0.1, 0.5, and 1 μ M compound, respectively. The coefficient of variation was less than 10%, and the actual concentration of the compounds ranged from 88 to 112.1%. All assay methods thus provide good accuracy and precision.

Calculation of $CL_{int, in vitro}$. $CL_{int, in vitro}$ values were calculated from the substrate disappearance rate in hepatic microsomes as follows. If substrate disappearance can be assumed to follow a first-order reaction, the unchanged drug profile as a function of time $[C(t)]$ is described as follows:

$$C(t) = C_0 \cdot \exp(-k_e \cdot t) \quad (1)$$

TABLE 1

Physiological parameters for calculation of intrinsic clearance in rats, dogs, and humans

Reference: Iwatsubo et al., 1997b, Davies and Morris, 1993

	Rat	Dog	Human
Microsomal protein per gram of liver (mg of protein/g of liver)	44.8	77.9	48.8
Liver weight per kilogram of body weight (g of liver/kg)	40	32	25.7
Liver blood flow (ml/min/kg)	55.2	30.9	20.7

where C_0 is initial concentration of the compound, and k_e is the disappearance rate constant of unchanged drug (per minute).

Furthermore, initial metabolic rate (V_0) per unit milligram of microsomal protein ($\mu\text{mol}/\text{min}/\text{mg}$ microsomal protein) is described by eq. 2.

$$V_0 = k_e \cdot C_0 / P_{MS} \quad (2)$$

where P_{MS} is the microsomal protein concentration (mg/ml).

On the other hand, from the Michaelis-Menten equation, V_0 is described by eq. 3.

$$V_0 = V_{\max} \cdot C_0 / (K_m + C_0) \quad (3)$$

If the substrate concentration used in the experiments (1 μM) is below the K_m for the P450-mediated reactions, the drug concentration may be assumed to be much smaller than K_m ($K_m \gg C_0$). Thus, V_0 can be expressed by eq. 4.

$$V_0 = V_{\max} / K_m \cdot C_0 \quad (4)$$

Consequently,

$$CL_{\text{int, in vitro}} = V_{\max} / K_m = V_0 / C_0 \quad (5)$$

$CL_{\text{int, in vitro}}$ was thus calculated by eq. 5 based on the time course of unchanged drug concentrations by least square linear regression. The $CL_{\text{int, in vitro}}$ values expressed per milligram of microsomal protein calculated from the in vitro metabolism experiments were expressed per kilogram of body weight by taking the microsomal protein content per gram liver and the liver weight per kilogram of body weight shown in Table 1 into consideration.

In Vivo Data. Sources of pharmacokinetic data. In vivo clearance under linear conditions, f_u and R_B data, were obtained from in house and literature. The in vivo pharmacokinetic data were considered to be reliable since the in vivo pharmacokinetic experiments were performed based on accurate methods and appropriate protocols. The in vivo clearance value was calculated by dividing the dose by the area under the plasma concentration curve. When the in vivo clearance value was not expressed per kilogram of body weight, this value was converted so that it was expressed per kilogram of body weight by taking the mean value of body weight in the literature or a body weight of 250 g, 10 kg, and 70 kg for rats, dogs and humans, respectively. The fraction absorbed from the intestinal tract (F_a) of FK1052, FK480, and zolpidem were calculated by summing the recoveries of radioactivity in bile and urine after oral administration of ^{14}C model compounds to rats. F_a of omeprazole, nifedipine, nilvadipine, diazepam, and diltiazem were estimated to be 1.0 from the literature data.

Calculation of $CL_{\text{int, in vitro}}$. CL_{H} values were determined from eq. 6 by use of the CL_{oral} values, except the CL_{H} for omeprazole in dogs, where only CL_{tot} data was available. CL_R was considered to be negligible for the model compounds.

$$CL_{\text{H}} = (CL_{\text{oral}} / R_B) \cdot (F_H \cdot F_a) \quad (6)$$

$CL_{\text{int, in vitro}}$ was calculated from the following equations using the well stirred, parallel-tube (Pang and Rowland, 1977), and dispersion models (Roberts and Rowland, 1986).

$$CL_{\text{H}} = Q_H \cdot (1 - F_H) \quad (7)$$

Well stirred model:

$$F_H = Q_H / (Q_H + (f_p / R_B) \cdot CL_{\text{int}}) \quad (8)$$

Parallel-tube model:

$$F_H = \exp\{-(f_p / R_B) \cdot CL_{\text{int}}\} \quad (9)$$

Dispersion model:

$$F_H = \frac{4a}{(1+a)^2 \exp\{(a-1)/2D_N\} - (1-a)^2 \exp\{-(a+1)/2D_N\}} \quad (10)$$

$$a = (1 + 4R_N \cdot D_N)^{1/2} \quad (11)$$

$$R_N = (f_p / R_B) \cdot CL_{\text{int}} / Q_H \quad (12)$$

$$D_N = 0.17 \quad (13)$$

$CL_{\text{int, in vivo}}$ for omeprazole in dogs was calculated from eqs. 7 to 13 by use of the CL_{tot} value, where CL_{H} is equal to CL_{tot} / R_B .

E_H were calculated from eq. 14.

$$E_H = 1 - F_H = CL_{\text{H}} / Q_H \quad (14)$$

Prediction of Human $CL_{\text{int, in vivo}}$, CL_{oral} , and E_H . Estimation of scaling factor. Values of scaling factor were estimated from the following equation:

$$\text{Scaling factor} = CL_{\text{int, in vivo}} / CL_{\text{int, in vitro}} \quad (15)$$

Prediction of human $CL_{\text{int, in vivo}}$. Human $CL_{\text{int, in vivo}}$ were predicted based on human $CL_{\text{int, in vitro}}$ using the following two methods: 1) disregarding animal scaling factor:

$$\text{Predicted human } CL_{\text{int, in vivo}} = \text{human } CL_{\text{int, in vitro}} \quad (16)$$

2) including animal scaling factor:

$$\text{Predicted human } CL_{\text{int, in vivo}} = \text{human } CL_{\text{int, in vitro}} \cdot \text{animal scaling factor} \quad (17)$$

For these $CL_{\text{int, in vivo}}$ predictions, success was assessed by the geometric mean of the ratio of predicted and actual values (Obach, 1999). Thus:

$$\text{Average fold error} = 10^{[\sum \log(\text{actual/predicted})] / n} \quad (18)$$

Prediction of in vivo human CL_{oral} and E_H . In vivo human CL_{oral} and E_H were predicted using eqs. 6 to 14, based on the human $CL_{\text{int, in vitro}}$ corrected with animal scaling factor.

Binding of Model Compounds to Microsomes. Determination of $f_{u, \text{microsome}}$. Model compounds (final concentration, 1 μM) were mixed with liver microsomes (at protein concentrations used for the respective metabolic incubations) in 100 mM phosphate buffer (pH 7.4) containing 10 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 5 mM MgCl_2 . The mixtures (1.2 ml) were delivered to one side of a dialysis cell containing a preconditioned dialysis membrane (molecular weight cut off, 10 kDa) (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). To the other side of the membrane was delivered 1.2 ml of the phosphate buffer containing glucose-6-phosphate and MgCl_2 . The cells were sealed, and the apparatus was incubated at 37°C for 4 h. After an incubation period, the microsome and buffer samples were removed and analyzed by HPLC. The HPLC conditions were the same as described above. The unbound fraction was calculated from the following:

$$f_{u, \text{microsome}} = \frac{\text{unchanged compound concentration in buffer side}}{\text{unchanged compound concentration in microsome side}} \quad (19)$$

Calculation of $CL_{\text{int, in vitro}}$ and the scaling factor. By incorporating the correction with the unbound fraction in the microsomal incubation mixture, $CL_{\text{int, in vitro}}$ is defined as (Iwatsubo et al., 1997a):

$$CL_{\text{int, in vitro}} = CL_{\text{int, in vitro}} / f_{u, \text{microsome}} \quad (20)$$

The value of scaling factor, which is the ratio of $CL_{\text{int, in vivo}}$ to $CL_{\text{int, in vitro}}$, was calculated from eq. 15

TABLE 2
Estimation of $CL_{int, in vivo}$ from in vivo pharmacokinetic data for the model compounds in rats, dogs, and humans

Compounds	Species	CL_{oral} or CL_{tot}	F_a	f_p	R_B	$CL_{int, in vivo}$			E_H	Reference
						Well Stirred	Parallel-Tube	Dispersion		
		ml/min/kg body weight				ml/min/kg body weight				
FK1052	Rat	875	0.95	0.03	1.21	27708.33	5785.60	7952.74	0.93	In house data
	Dog	62.7		0.017	0.82	3503.82	1802.28	2114.04	0.70	
	Human	26.4		0.016	0.78	1567.50	945.95	1071.18	0.61	
FK480	Rat	81.1	0.70	0.01	0.626	5677.00	3358.32	3824.30	0.62	In house data
	Dog	6.6		0.006	0.6	770.00	687.51	709.16	0.20	
	Human	2.4		0.005	0.6	336.00	315.14	320.79	0.12	
Zolpidem	Rat	106	0.89	0.13	0.89	725.69	405.00	466.88	0.66	Ishibashi et al. (1993a,b)
	Human	7.1		0.04	0.66	157.98	129.84	137.31	0.32	
Omeprazole	Rat	710	1.00	0.125	0.78	5680.00	985.69	1393.01	0.94	Watanabe et al. (1994)
	Dog	6.3 ^a		0.097	0.60	98.38	79.36	84.03	0.34	
	Human	22.2		0.042	0.62	528.57	306.86	350.73	0.63	
Nicardipine	Rat	1212	1.00	0.084	1.0 ^b	14428.57	2059.22	2992.55	0.96	Higuchi and Shiobara (1980a,b)
	Dog	597		0.062	1.0 ^b	9629.03	1500.95	2154.28	0.95	
	Human	131		0.068	1.0 ^b	1926.47	606.32	779.70	0.86	
Nilvadipine	Rat	2852	1.00	0.0117	0.922	243760.68	17589.94	27932.16	0.98	Tokuma et al. (1987, 1988)
	Dog	36		0.008	0.836	4500.00	2818.31	3170.52	0.58	
	Human	107		0.013	0.789	8230.77	2539.97	3277.20	0.87	
Diazepam	Rat	103.8	1.00	0.137	1.04	757.66	432.66	496.31	0.64	Diaz-Garcia et al. (1992); Luurila et al. (1996)
	Human	0.7		0.032	1.04	21.88	21.53	21.53	0.03	
										(1985); Klotz et al. (1976)
Diltiazem	Rat	300.2	1.00	0.184	0.93	1631.52	536.77	684.98	0.85	Tsui et al. (1994); Etoh et al. (1983)
	Dog	201.6		0.298	1.0 ^b	676.54	209.27	269.91	0.87	
	Human	64.2		0.22	1.0	291.82	132.79	159.59	0.76	

^a CL_{tot}

^b Assumed value.

Results

In Vivo Pharmacokinetic Data of Model Compounds. In vivo pharmacokinetic data for the model compounds are summarized in Table 2. F_a values were high, in the range of 0.7 to 1.0. The values of unbound fraction in plasma (or serum) (f_p) were relatively low for all compounds ranging from the highest value for diltiazem (f_p : rat, 0.184; dog, 0.298; human, 0.22) to the lowest value for FK480 (f_p : rat, 0.01; dog, 0.006; human, 0.005). In vivo clearance, $CL_{int, in vivo}$, and E_H values differed markedly among the different species for each compound. Omeprazole, for example, is characterized by a high E_H (0.94) in rats. An intermediate E_H value (0.63) was observed in humans, whereas dogs exhibited a low E_H (0.34). In vivo human E_H ranged widely from 0.03 for diazepam to 0.87 for nilvadipine among the model compounds.

In Vitro Metabolism in Microsomes and Estimation of Scaling Factor. Figure 2 illustrates the time courses of the unchanged model compounds in microsomes. The unchanged drug profiles at 1 μ M showed that the linear log concentration declines so that the metabolism follows first-order reaction under this condition. Large interspecies differences were also observed in $CL_{int, in vitro}$ for the model compounds. Table 3 shows the human $CL_{int, in vitro}$ calculated from the time courses of the model compounds in microsomes and the values of human scaling factor, which are the ratios of $CL_{int, in vitro}$ obtained from in vivo pharmacokinetic data to $CL_{int, in vitro}$. The human scaling factor values calculated using the well stirred model were about 26.6-fold for FK1052, about 5-fold for FK480, zolpidem, omeprazole, and nilvadipine, and 1- to 2.5-fold for nicardipine, diazepam, and diltiazem, showing marked difference among the model compounds. In the same way, the human scaling factor values calculated using the parallel-tube and the dispersion models were 0.3- to 16.1-fold and 0.4- to 18.2-fold, respectively, showing marked difference among the model compounds.

Figure 3 shows the differences in scaling factor between animals and humans. Most values of animal scaling factor were within 2-fold of the values in humans, except that the animal scaling factors for

FK480 were 3.5- to 5.4-fold larger than that in humans. The results do not depend on the kind of the mathematical models and animal species.

Prediction of Human $CL_{int, in vivo}$, CL_{oral} and E_H by Use of Animal Scaling Factor. Human $CL_{int, in vitro}$ values corrected both with and without animal scaling factor are plotted versus actual $CL_{int, in vivo}$ values calculated using the mathematical models in Fig. 4. Without animal scaling factor consideration, only two (the well stirred model) or three (the parallel-tube and the dispersion models) of eight prediction values were within 2-fold of the actual $CL_{int, in vivo}$, resulting in a large underestimation for most of the compounds. By contrast, using human $CL_{int, in vitro}$ corrected with rat and/or dog scaling factor, 9 (the parallel-tube model) or 10 (the well stirred and the dispersion models) of 13 prediction values were within 2-fold of the actual $CL_{int, in vivo}$, significantly improving the predictability of $CL_{int, in vivo}$.

The geometric mean accuracy values as indices of human $CL_{int, in vitro}$ predictability are listed in Table 4. The value of one means perfect predictability, and the poorer becomes predictability with the larger values. The geometric mean accuracy values without animal scaling factor consideration were 3 to 4. By contrast, the predictability was substantially improved with the geometric mean accuracy value of less than 2.

Furthermore, human in vivo CL_{oral} and E_H values were predicted from human $CL_{int, in vitro}$ corrected with animal scaling factor. Figure 5 presents comparisons of the predicted CL_{oral} values with the observed CL_{oral} . The CL_{oral} values predicted using the well stirred model were in good agreement with the observed values. When CL_{oral} was predicted using the parallel-tube and the dispersion models, good correlations were observed, with the exception of the overestimation for nilvadipine.

Figure 6 shows the relation between $CL_{int, in vitro}$ and in vivo E_H . The predicted E_H based on $f_b \cdot CL_{int, in vitro}$ corrected with animal scaling factor was close to the observed E_H for all mathematical models. This result suggests that the human $CL_{int, in vitro}$ with animal

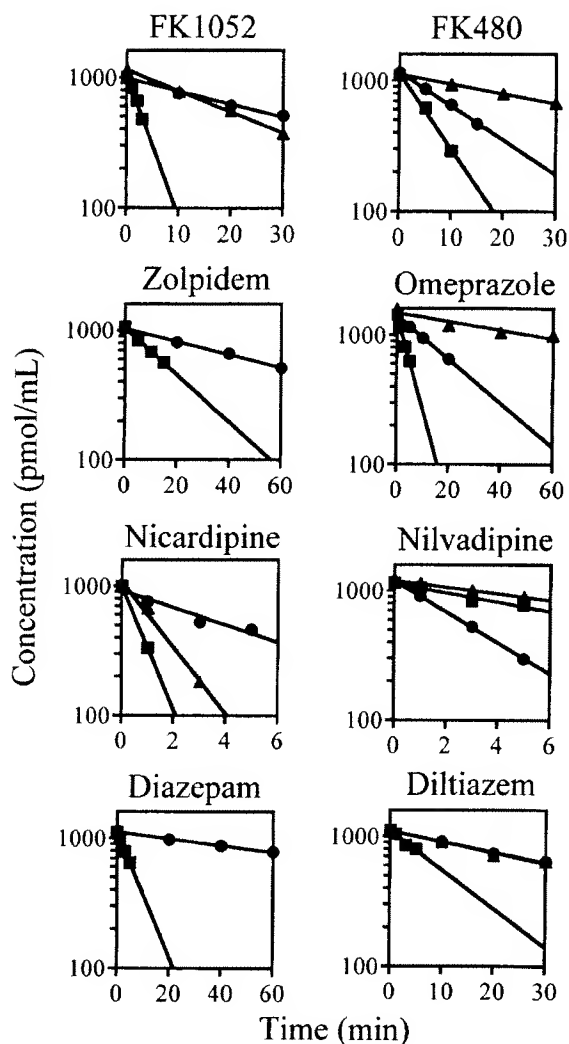


FIG. 2. Time courses of unchanged compounds in microsomes.

Each compound (at a concentration 1 μ M) was incubated for various time periods at 37°C in rat, dog, and human liver microsomes. Microsomal protein concentrations used were 0.2 mg/ml (nicardipine, nilvadipine, and diltiazem), 0.5 mg/ml (FK1052, zolpidem, omeprazole, and diazepam), and 1.0 mg/ml (FK480). ■, rat liver microsomes; ▲, dog liver microsomes; ●, human liver microsomes. The solid lines represent the linear regression lines by the least-squares method.

TABLE 3

Estimation of human $CL_{int, in vitro}$ in hepatic microsomes and their values of scaling factor for the model compounds

Compounds	$CL_{int, in vitro}$ ml/min/kg body weight	Scaling Factor ($CL_{int, in vivo}/CL_{int, in vitro}$)		
		Well Stirred	Parallel-Tube	Dispersion
FK1052	58.90	26.6	16.1	18.2
FK480	74.21	4.5	4.2	4.3
Zolpidem	29.00	5.4	4.5	4.7
Omeprazole	98.08	5.4	3.1	3.6
Nicardipine	1736.34	1.1	0.3	0.4
Nilvadipine	1712.88	4.8	1.5	1.9
Diazepam	15.00	1.5	1.4	1.4
Diltiazem	118.15	2.5	1.1	1.4

scaling factor consideration could give a good prediction of E_H and hepatic clearance in humans.

Binding of Model Compounds to Microsomes. Binding experi-

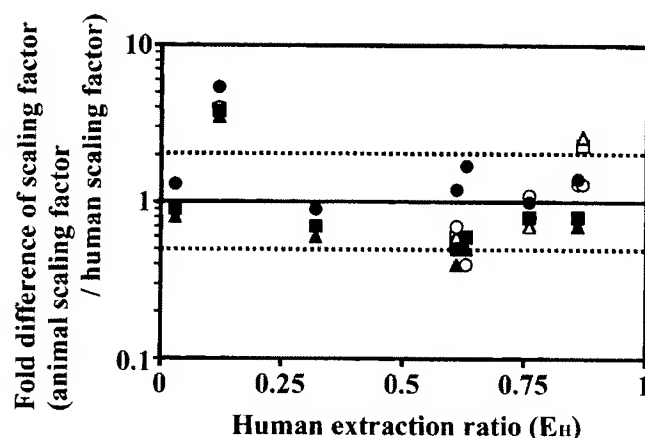


FIG. 3. Differences in scaling factor between animals and humans.

Symbols represent the fold differences of scaling factor calculated using the well stirred model (○), the parallel-tube model (△), and the dispersion model (□). Closed symbols represent the fold differences of scaling factor between rats and humans. Open symbols represent the fold differences of scaling factor between dogs and humans. The solid line represents the line of unity. The area between the dotted lines represents an area within 2-fold error.

ments were conducted in similar conditions used in *in vitro* microsomal metabolism studies but were conducted in the absence of NADP⁺ so that metabolism of the compounds would not occur. Recovery of the compounds was more than 90%, except nicardipine where the recovery was about 50%. Table 5 summarizes the unbound fraction in liver microsomes, human $CL_{int, in vitro}$ corrected with $f_{u, microsome}$, and the scaling factor values. $f_{u, microsome}$ of diltiazem in rats could not be estimated because this compound was metabolized by microsomal esterase (see *Materials and Methods*). Human $f_{u, microsome}$ values were dependent on the model compounds. For example, although FK1052, FK480, nicardipine, and nilvadipine were highly bound to microsome with free fraction values ranging from 0.112 to 0.443, zolpidem, omeprazole, diazepam, and diltiazem showed low binding with free fraction values ranging from 0.745 to 0.975. $f_{u, microsome}$ of each compound to rat and dog liver microsomes was similar to that measured in human liver microsomes. For FK1052, FK480, and nilvadipine, the human scaling factor values obtained by correcting human $CL_{int, in vitro}$ with $f_{u, microsome}$ approached unity. However, the scaling factor for FK1052 were still 5.2- to 8.6-fold. In the case of nicardipine, these values by correcting human $CL_{int, in vitro}$ with $f_{u, microsome}$ became smaller than unity (0.05–0.1-fold). For zolpidem, omeprazole, diazepam, and diltiazem, corrections of $CL_{int, in vitro}$ with $f_{u, microsome}$ corrections did not change the human scaling factor values significantly. Consequently, the scaling factor for zolpidem and omeprazole remained 4.2- to 5.1-fold and 3.1- to 5.3-fold, respectively.

Discussion

In the present study, we investigated the quantitative prediction of human hepatic metabolic clearance from *in vitro* experiments using liver microsomes focusing on P450 metabolism. The values of $CL_{int, in vitro}$ and $CL_{int, in vivo}$ for each model compound were compared in rats and/or dogs, and humans. As a result, 1) scaling factor values ($CL_{int, in vivo}/CL_{int, in vitro}$) were similar in the different animal species; 2) scaling factor values were different in each compound; 3) successful predictions of human $CL_{int, in vivo}$ were obtained by considering animal scaling factor; and 4) use of human $CL_{int, in vitro}$ corrected with animal scaling factor gave good predictions of CL_{oral} and E_H in humans. Namely, the empirical prediction method is a simple one of incorporating additional information (which is compound specific)

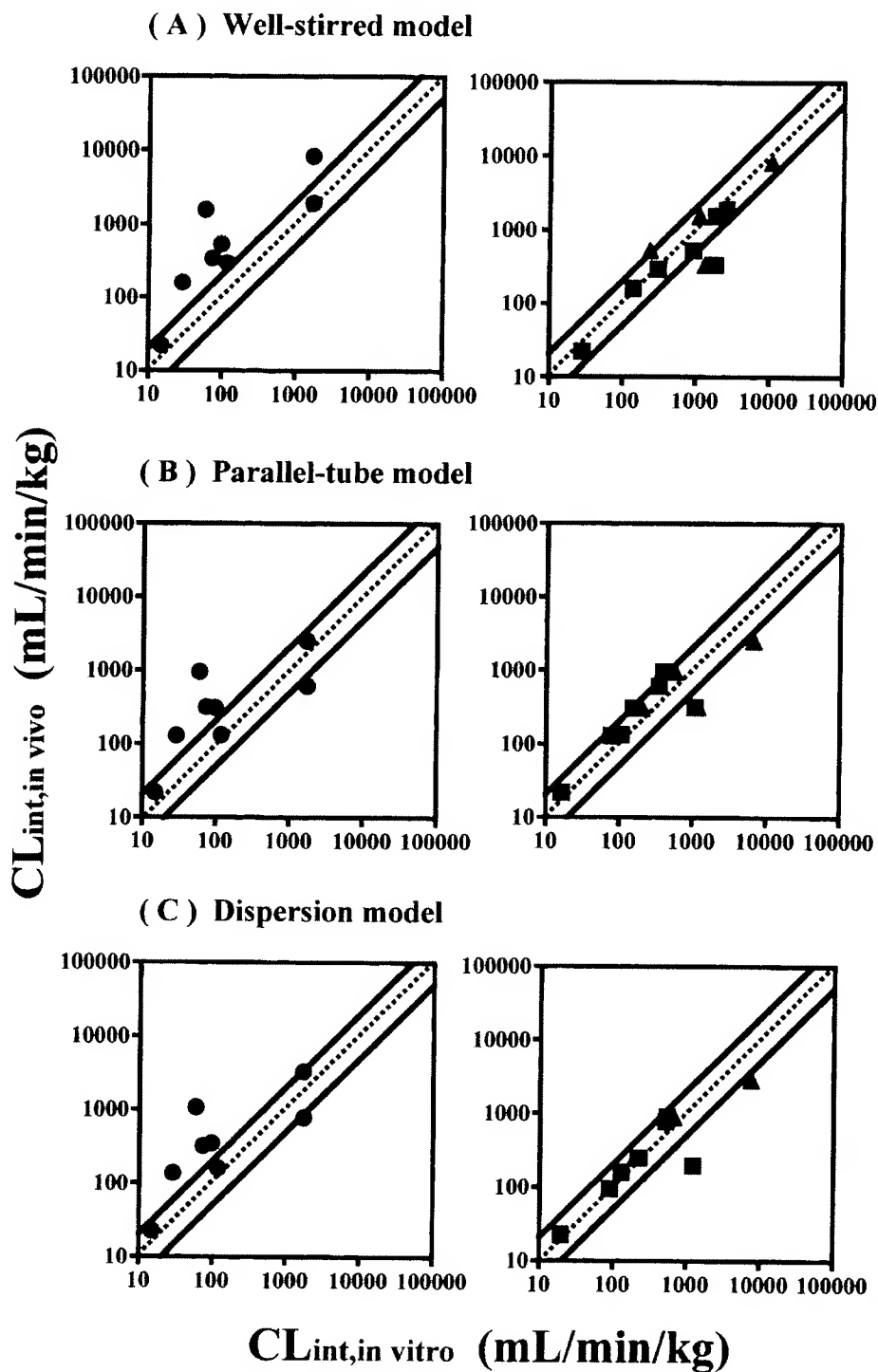


FIG. 4. Comparison of predicted $CL_{int,in vitro}$ with $CL_{int,in vivo}$ in humans calculated using the well stirred model (A), the parallel-tube model (B), and the dispersion model (C).

●, human $CL_{int,in vitro}$ not corrected by animal scaling factor using eq. 16; ■, human $CL_{int,in vitro}$ corrected by rat scaling factor using eq. 17; ▲, human $CL_{int,in vitro}$ corrected by dog scaling factor using eq. 17. The dotted lines represent the lines of unity. The area between the solid lines represents an area within 2-fold error.

from animal studies and is based on the assumption that any in vitro-in vivo difference seen in humans is also apparent in animals to approximately the same degree.

In a conventional method, $CL_{int,in vitro}$ is obtained from K_m and V_{max} , which are estimated by measuring the production of metabolites over a wide range of drug concentrations. On the other hand,

$CL_{int,in vitro}$ in the proposed method is obtained from substrate disappearance rate at a single drug concentration. From comparison of these methods, advantages of the proposed method are raised as follows: 1) simple to conduct; 2) can be done for many compounds; 3) metabolites do not need to be known; 4) can be easily done without radiolabel; and 5) can yield enzyme kinetic data based on the disap-

TABLE 4

Accuracy of human $CL_{int, in vivo}$ prediction methods

The n values refer to the number of predicted values of $CL_{int, in vivo}$ ($CL_{int, predicted}$) that were compared with the actual human $CL_{int, in vivo}$ obtained from in vivo pharmacokinetic data ($CL_{int, actual}$).

Methods	n	Average Fold Error ($CL_{int, actual}/CL_{int, predicted}$)		
		Well Stirred	Parallel-Tube	Dispersion
Without scaling factor	8	4.02	3.00	3.20
With rat scaling factor	7	1.57	1.85	1.67
With dog scaling factor	6	1.68	2.00	1.88

pearance of parent compounds. On the contrary, disadvantages of the proposed method are as follows: 1) it is difficult to measure very low $CL_{int, in vitro}$ values; 2) does not get individual metabolite information; 3) and does not obtain K_m and V_{max} parameters. Recent studies have also calculated $CL_{int, in vitro}$ from substrate disappearance (Lave et al., 1997; Obach, 1999).

In this study, we assumed that extrahepatic clearances could be negligible. Recently, it has been reported that the first-pass metabolism in human small intestine is not negligible for some drugs, such as cyclosporine, which is metabolized mainly by CYP3A4 (Benet et al., 1996). Of the model compounds, FK480 (in house data), zolpidem (Pichard et al., 1995), nicardipine (Guengerich et al., 1991), nilvadipine (in house data), and diltiazem (Sutton et al., 1997) are metabo-

lized mainly by CYP3A4. Human $CL_{int, in vivo}$ of these compounds were calculated from the CL_{oral} after oral administration. Nevertheless, the $CL_{int, in vivo}$ values predicted from human $CL_{int, in vitro}$ with animal scaling factor consideration were comparable with the observed values (Fig. 4). From this result, although the possibility of intestinal metabolism of the model compounds cannot be completely excluded, it may be reasonable to consider the metabolism of these compounds mainly in the liver for predicting $CL_{int, in vivo}$. In the future, we would evaluate in vitro-in vivo scaling for the model compounds obviously metabolized in small intestine.

When $CL_{int, in vivo}$ was calculated from in vivo clearance data, or when CL_H and E_H were calculated from $CL_{int, in vitro}$, three frequent mathematical models (the well stirred, parallel-tube, and dispersion models) were used. Although there were a few drugs of which the human scaling factor values of all mathematical models were close to unity (nicardipine, diazepam, and diltiazem), the values for some drugs (FK1052, FK480, zolpidem, and omeprazole) were 3.1- to 26.6-fold (Table 3), resulting in $CL_{int, in vivo}$ values larger than the $CL_{int, in vitro}$ values. These findings suggest that the conventional prediction method, which directly applies $CL_{int, in vitro}$ into the mathematical models, cannot always predict in vivo clearance for all compounds. By contrast, the proposed method, considering the scaling factor, yielded more accurate prediction of human $CL_{int, in vivo}$ that was mostly within 2-fold of actual values (Fig. 4).

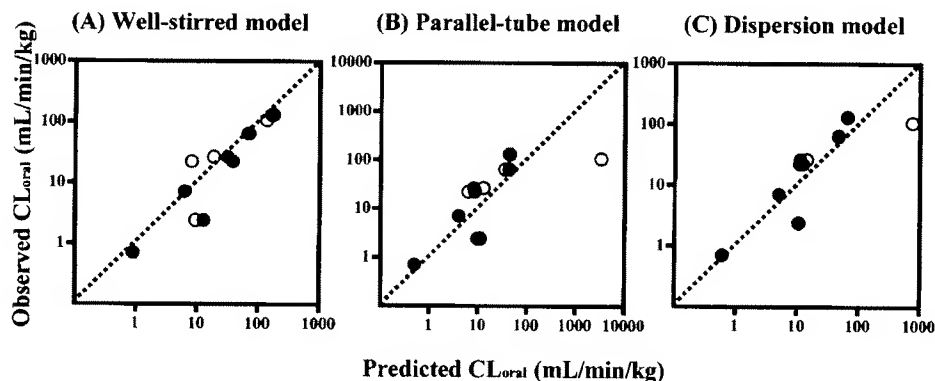


FIG. 5. Comparison of predicted CL_{oral} with observed CL_{oral} in humans calculated using the well stirred model (A), the parallel-tube model (B), and the dispersion model (C).

●, predicted CL_{oral} calculated using human $CL_{int, in vitro}$ corrected by rat scaling factor; ○, predicted CL_{oral} calculated using human $CL_{int, in vitro}$ corrected by dog scaling factor. The dotted lines represent the lines of unity.

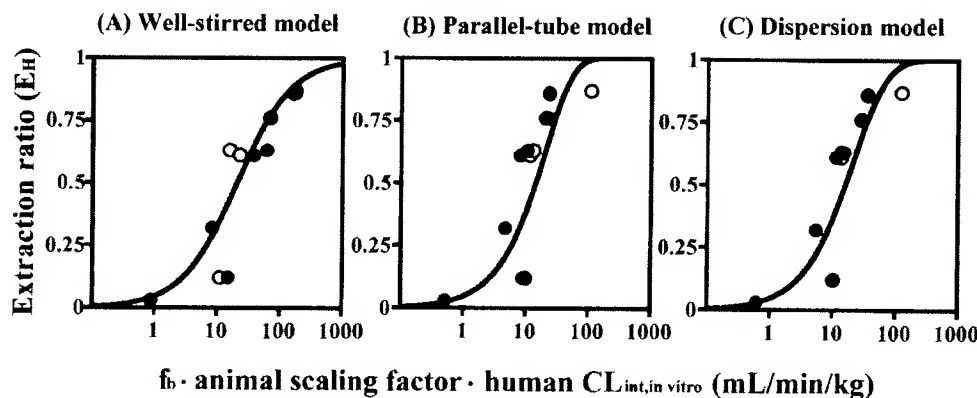


FIG. 6. Correlation between $CL_{int, in vitro}$ and in vivo E_H in humans calculated using the well stirred model (A), the parallel-tube model (B), and the dispersion model (C).

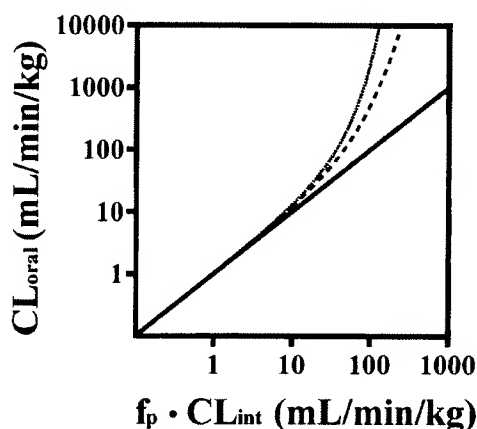
●, human $f_b \cdot CL_{int, in vitro}$ corrected by rat scaling factor; ○, human $f_b \cdot CL_{int, in vitro}$ corrected by dog scaling factor. The solid lines represent the simulated curves based on the mathematical models.

TABLE 5

Binding of the model compounds to hepatic microsomes and their effects on predicting human $CL_{int, in vivo}$

Compounds	Microsomal Protein Concentration mg/ml	$f_{u, \text{microsome}}$			Human $CL_{int, in vitro}$ ml/min/kg	Human Scaling Factor		
		Rat	Dog	Human		Well Stirred	Parallel-Tube	Dispersion
FK1052	0.5	0.347	0.334	0.323	182.35	8.6	5.2	5.9
FK480	1.0	0.119	0.120	0.112	662.59	0.5	0.5	0.5
Zolpidem	0.5	0.938	0.940	0.939	30.88	5.1	4.2	4.4
Omeprazole	0.5	0.929	0.972	0.975	100.59	5.3	3.1	3.5
Nicardipine	0.2	0.175	0.183	0.129	13460	0.1	0.05	0.06
Nilvadipine	0.2	0.469	0.470	0.443	3866.55	2.1	0.7	0.8
Diazepam	0.5	0.781	0.752	0.745	20.13	1.1	1.1	1.1
Diltiazem	0.2	N.D.	0.907	0.863	136.91	2.1	1.0	1.2

N.D., not determined.

FIG. 7. Correlation between CL_{int} and CL_{oral} .

The solid line is the simulated curve using eqs. 6 to 8, based on the well stirred model. The dotted line is the simulated curve using eqs. 6, 7, and 9, based on the parallel-tube model. The broken line is the simulated curve using eqs. 6, 7, and 10 to 13, based on the dispersion model.

Presently, it is not clear why each compound has a intrinsic scaling factor. However, there are two possible reasons below. First, $f_{u, \text{microsome}}$ in the reaction mixture may have an influence on the $CL_{int, in vitro}$ value. Obach (1999) has examined $f_{u, \text{microsome}}$ of model compounds and the potential impact that such binding has on the prediction of in vivo clearance from $CL_{int, in vitro}$ for these compounds. Those results showed that incorporation of $f_{u, \text{microsome}}$ generally yielded more accurate predictions of human clearance. In the same way, we have evaluated $f_{u, \text{microsome}}$ of the model compounds and the change of the scaling factor values when correcting with $f_{u, \text{microsome}}$. However, $CL_{int, in vitro}$, when by correcting with $f_{u, \text{microsome}}$, were not in agreement with the $CL_{int, in vivo}$ for some compounds; the scaling factor values for FK1052, zolpidem, and omeprazole were several-fold, and the scaling factor values for nicardipine were much smaller than unity (Table 5). This suggests that the scaling factor different from unity might not be due only to the drug binding to liver microsomes. The second reason may be related to some assumptions of the mathematical models below: 1) the distribution of drug into the liver is assumed to be perfusion-rate limited, and diffusion of drug into hepatocytes is rapid and not subject to any diffusional barriers; 2) it is assumed that only the free (unbound to macromolecules in blood) drug crosses the cell membrane and subsequently occupies the enzyme site; and 3) a homogeneous distribution of drug-metabolizing enzymes within the liver acinus is adopted (Houston and Carlile, 1997). If any assumptions are incorrect, the discrepancies between $CL_{int, in vivo}$ and $CL_{int, in vitro}$ would be observed.

The use of hepatic microsomes to predict in vivo CL_H requires acceptance of some assumptions (Obach, 1999). First, oxidative metabolism predominates over other metabolic routes, such as direct conjugation, reduction, hydrolysis, etc. Second, rates of metabolism measured using animal and human microsomes in vitro are truly reflective of these exist in vivo. It has been reported that diltiazem is metabolized in part by hepatic microsomal esterase in rats (LeBoeuf and Bélanger, 1987). Therefore, the $CL_{int, in vitro}$ of microsomal esterase was estimated in conditions where no NADPH-generating system was included in the incubation mixture. As a result, the $CL_{int, in vitro}$ of microsomal esterase represented approximately 30% of the $CL_{int, in vitro}$ observed in the presence of NADPH-generating system in rats, whereas the $CL_{int, in vitro}$ of microsomal esterase in dogs and humans were hardly observed (data not shown). Even for diltiazem, scaling factor values were similar among the different species (Table 3), suggesting that the $CL_{int, in vitro}$, which was measured in conditions with NADPH-generating in rats, reflects the metabolic rates by both P450 and esterase. However, future studies should also focus on evaluating in vitro-in vivo scaling for the other compounds metabolized by microsomal esterase to clarify the validity of the consideration. Third, the substrate concentration used ($1 \mu\text{M}$ in this study) is well below the apparent K_m . It may be necessary to confirm this assumption. For example, for only FK480, 3.5- to 5.4-fold differences in scaling factor between animals and humans were observed (Fig. 3). It may be accounted for by the possibility that the animal $CL_{int, in vitro}$ could be seriously underestimated because of the lower K_m value at $1 \mu\text{M}$. However, the $CL_{int, in vitro}$ at several concentrations below $1 \mu\text{M}$ were similar to that at $1 \mu\text{M}$ in animals and humans (data not shown). Consequently, it was considered that the $CL_{int, in vitro}$ at $1 \mu\text{M}$ was under linear condition.

$CL_{int, in vitro}$ corrected with animal scaling factor could give a good prediction of in vivo CL_{oral} and E_H in humans. But, for a high-clearance drug, nilvadipine, the CL_{oral} values using the parallel-tube and dispersion models were overestimated (Fig. 5). The reason is seen in the well stirred model where CL_{oral} is in proportion to $CL_{int, in vitro}$, whereas the parallel-tube and dispersion models give exponential correlation between $CL_{int, in vitro}$ and CL_{oral} for high-clearance drugs (Fig. 7). As a result, the error of predicted CL_{oral} , which is caused from the error of predicted $CL_{int, in vitro}$, is magnified in the parallel-tube and dispersion models compared with the error in the well stirred model. Also, in the oral case, $CL_{int, in vitro}$ calculated based on the parallel-tube and dispersion models was affected to some extent by Q_H for the high-clearance drugs, whereas $CL_{int, in vitro}$ calculated based on the well stirred model was not affected (Iwatsubo et al., 1997a). In the case of predicting CL_{oral} for high-clearance drugs, it may be necessary to consider the selection of the mathematical models.

In conclusion, we investigated a new variant method on the quan-

titative prediction of human hepatic clearance from in vitro experiments, focusing on P450 metabolism with eight model compounds. Successful predictions of human hepatic clearance were obtained by use of the human $CL_{int,in vitro}$ corrected with animal scaling factors, which are the ratios of $CL_{int,in vivo}$ to $CL_{int,in vitro}$. This method would provide more reliable prediction of human hepatic clearance and be useful in drug discovery.

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EXHIBIT

20

Exhibit 20

Protocol for Liver Microsome Assay

Compounds were briefly incubated at a final concentration of 1 μ M in the presence of human liver microsomes (XenoTech, LLC, Lenexa, Kansas) (0.5 mg protein/mL) in Tris buffer (100 mM, pH 7.4 at 37°C). Following a five-minute pre-incubation period, enzymatic reaction was initiated by addition of NADPH to a final concentration 2.0 mM, and samples were collected at 0, 5, 10, and 30 minutes. The concentration of residual compound was analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS).

EXHIBIT

21

TABLE III: Intrinsic Clearance in Human Microsomes

Example No.	Salt Form	Intrinsic Clearance, CL _{INT} (μ l/min/mg)
Example A	Tartrate	7
Example B	HCl	2
Example 46	HCl	47
Example 58	HCl	5
Example 59	HCl	26
Example 60	HCl	8
Example 72	HCl	39
Example 78	HCl	35
Example 85	HCl	34
Example 110	Oxalate	8.5
Example 112	HCl	15
Example 120	HCl	17
Example 127	HCl	3
Example 131	HCl	15
Example 132	HCl	14
Example 135	HCl	26
Example 136	HCl	20
Example P-1 ¹	HCl	31
Example P-2	HCl	32.5
Example P-3	HCl	34.5

¹ Refers to compounds recited in Andersson at pages 25-27.